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# Fibronectin-dependent Activation of CaMK-II Promotes Focal Adhesion Turnover by Inducing Tyrosine Dephosphorylation of FAK and Paxillin

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FIBRONECTIN-DEPENDENT ACTIVATION OF CAMK-II PROMOTES FOCAL  
ADHESION DISASSEMBLY BY INDUCING TYROSINE DEPHOSPHORYLATION  
OF FAK AND PAXILLIN

A Dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy at Virginia Commonwealth University.

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## List of Abbreviations

$\alpha$	Alpha
Ala	Alanine
Asp	Aspartate
ATP	Adenosine Triphosphate
$\beta$	Beta
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
$\gamma$	Gamma
$\delta$	Delta
c-abl	Ableson 1 tyrosine kinase
$\text{Ca}^{2+}$	Calcium ion
CaMK-I	Calcium/Calmodulin-dependent Protein Kinase Type I
CaMK-II	Calcium/Calmodulin-dependent Protein Kinase Type II
CaMK-IV	Calcium/Calmodulin-dependent Protein Kinase Type IV
CaM	Calmodulin
CHO	Chinese Hamster Ovary
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)] tetra-acetic acid
FAK	Focal adhesion kinase
FN	Fibronectin
Gab1	Grb2 associated binder 1
GFP	Green Fluorescent Protein
HEPES	(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HERG	Human Ether-a-go-go
HME	Human Mammary Epithelial
ICAP-1 $\alpha$	Integrin cytosolic domain associated protein 1 alpha
IgG	Immunoglobulin
IP <sub>3</sub>	Inositol 1, 4, 5-Trisphosphate
KN-93	2-[N-(2-Hydroxyethyl)-N-4-methoxybenzenesulfonyl]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine
Myr-AIP	Myristoylated autoinhibitory peptide
NBT	Nitro-blue tetrazolium
PBS	Phosphate-buffered saline
PIP <sub>2</sub>	Phosphatidylinositol-4, 5-bisphosphate
RAD	Arginine, alanine, aspartate
RGD	Arginine, glycine, aspartate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine

SHP-2	SH2 domain-containing tyrosine phosphatase type 2
TBS	Tris-buffered saline
TBSTA	Tris-buffered saline with Tween-20 and sodium azide
Thr	Threonine
TIRF	Total internal reflection fluorescence
Tween-20	Polyoxyethylene sorbitan monolaurate
Tyr	Tyrosine
VSM	Vascular smooth muscle

## Abstract

### FIBRONECTIN-DEPENDENT ACTIVATION OF CAMK-II PROMOTES FOCAL ADHESION DISASSEMBLY BY INDUCING TYROSINE DEPHOSPHORYLATION OF FAK AND PAXILLIN

By Charles Allen Easley, IV, Ph.D. Biochemistry and Molecular Biology

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor  
of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

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Transient elevations in  $\text{Ca}^{2+}$  have previously been shown to promote focal adhesion disassembly and cell motility. Yet the targets of these  $\text{Ca}^{2+}$  transients have not been fully examined. In this study, we demonstrate that CaMK-II, a  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase, is activated in response to  $\beta 1$  integrin engagement with fibronectin to influence fibroblast adhesion and motility. We also show that CaMK-II is dynamically localized to the cell surface using Total Internal Reflection Fluorescence microscopy

(TIRFm) and that inhibition of CaMK-II with two mechanistically distinct, membrane permeant inhibitors accelerates spreading on fibronectin, enlarges paxillin-containing focal adhesions and blocks cell motility. On the other hand, expression of constitutively active CaMK-II reduces cell attachment, eliminates paxillin from focal adhesions and decreases the phospho-tyrosine levels of both FAK and paxillin. Cell spreading, paxillin incorporation into focal adhesions and phospho-tyrosine levels of FAK and paxillin are restored when cells expressing constitutively active CaMK-II are subsequently treated with myr-AIP, a specific CaMK-II catalytic inhibitor. Like CaMK-II inhibition, constitutively active CaMK-II blocks cell motility. Thus, both CaMK-II inhibition and constitutive activation block cell motility through over-stabilization or destabilization of focal adhesions, respectively. These findings provide the first direct evidence that CaMK-II promotes focal adhesion turnover and thus enables cell motility by stimulating tyrosine dephosphorylation of focal adhesion proteins.

## General Introduction

### *Calcium/calmodulin-dependent protein kinase type II*

CaMK-II, a  $\text{Ca}^{2+}$ /calmodulin-dependent kinase, is a ubiquitously expressed, multi-purpose serine/threonine kinase that regulates numerous cellular functions ranging from cell cycle progression to neurite outgrowth in nascent axons (Easley et al. 2006; Faison et al. 2002; Fink et al. 2003; Lin et al. 2004; Morris et al. 1998; Rasmussen and Rasmussen 1994; Rasmussen and Rasmussen 1995; Shen et al. 1998; Tombes et al. 1995). In all metazoans, CaMK-II is encoded by up to four genes ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ), which can be alternatively spliced to form over three dozen different splice variants (Tombes et al. 2003). In its native state, CaMK-II forms a dodecameric structure with each subunit possessing a relative mass ( $M_r$ ) of 50-60 kDa. Each subunit consists of 4 major domains: catalytic, regulatory, variable and oligomerization domains (see appendix figure 1B) (Kolodziej et al. 2000; Tombes et al. 2003). Differences in CaMK-II splice variant localization and function are primarily attributed to alternative splicing sites within the variable domain (Tombes et al. 2003). The C-terminal oligomerization domain comprises the core of the dodecameric complex, with each N-terminal catalytic domain facing outward (see appendix figure 1A). This orientation upon assembly gives the native CaMK-II protein complex the appearance of a bicycle wheel, with the oligomeric center serving as the core and the catalytic subunits resembling spokes.

Upon signaling events that elevate cytosolic  $\text{Ca}^{2+}$  levels,  $\text{Ca}^{2+}$  binds to calmodulin. Activated calmodulin then binds to the regulatory domain on CaMK-II (sequence,

RRKLKGAILTTML) (Schulman et al. 1992). The conformational change that results from calmodulin binding relieves the tonic inhibition of the autoinhibitory domain (sequence, KKALRRQETVDAL) (Schulman et al. 1992). The autoinhibitory domain of one subunit is then exposed to the active site of the adjacent subunit in the dodecameric holoenzyme (Schulman et al. 1992). The autophosphorylation of the threonine (Thr) within the autoinhibitory domain (Thr<sup>286</sup> in  $\alpha$ , Thr<sup>287</sup> in  $\beta$ ,  $\gamma$ , and  $\delta$ ) confers Ca<sup>2+</sup> independence and allows CaMK-II to regulate cellular processes well after the re-uptake of cytosolic Ca<sup>2+</sup>.

While multiple CaMK-II isoforms exist and are involved in a variety of cellular functions, this study focuses on  $\delta_c$ , the simplest cytosolic CaMK-II variant that is the major isoform expressed in NIH/3T3 cells. Recent work in our lab has shown the CaMK-II variants from different genes can compensate for one another suggesting that the catalytic domains in each gene product is functionally identical. Moreover, these catalytic similarities appear to be evolutionarily conserved as mouse  $\delta_c$  CaMK-II can be used to rescue heart development in a  $\beta$  CaMK-II-depleted zebrafish embryo.

### ***$\beta 1$ Integrin***

Integrin proteins have been implicated in a number of biological processes, which include embryogenesis, skin regeneration, muscle and cartilage formation, immune surveillance and cancer cell metastasis (Brakebusch and Fassler 2005). The integrin family of proteins are heterodimeric transmembrane receptors consisting of both an  $\alpha$  and  $\beta$  subunit (Hynes 2002). Currently there are 18 known  $\alpha$ 's and 8  $\beta$ 's, which combine to form



24 different receptors (Hynes 2002).  $\beta 1$  represents the largest family of heterodimeric integrin receptors and are involved in 12 different ligand binding properties, all of which bind to components of the extracellular matrix (Brakebusch and Fassler 2005; Hynes 2002). In neurons,  $\alpha 6\beta 1$  and  $\alpha 7\beta 1$  bind laminin to induce attachment (Brakebusch and Fassler 2005). In fibroblasts, the primary integrin heterodimer,  $\alpha 5\beta 1$ , binds to fibronectin.

Upon engagement with components of the extracellular matrix, integrin receptors associate with the actin cytoskeleton through the formation of protein complexes on the  $\beta 1$  cytoplasmic tail known as focal adhesions. Focal adhesions are critical signaling and structural hubs found in migrating cells and are composed of dozens of proteins including talin, vinculin, focal adhesion kinase (FAK) and paxillin (Huttenlocher et al. 1995; Lauffenburger and Horwitz 1996; Ridley et al. 2003).

### ***Talin***

Talin is a 270 kDa anti-parallel homodimeric protein that is a major component of focal adhesions (Critchley 2004; Nayal et al. 2004). Talin contains a globular head domain that binds integrin, FAK, PIPKI $\gamma$ , and actin. The tail domain of talin contains additional binding sites for integrin and actin and also vinculin (Critchley 2004; Ling et al. 2002). To initiate talin association with integrin, the FAK/src tyrosine kinase complex activates PIPKI $\gamma$  and mediates its binding to talin. PIPKI $\gamma$  then locally generates phosphatidylinositol (4,5) biphosphate (PIP $_2$ ) (Ling et al. 2002; Ling et al. 2003), which transiently associates with talin to increase its binding affinity for integrin (Critchley 2004; Ling et al. 2003). Talin association with integrin increases the receptor's affinity for

components of the extracellular matrix, a process termed inside-out signaling (Critchley 2004; Ling et al. 2003).

During cell motility, talin is highly regulated at the trailing edge of the cell, where focal adhesion breakdown is necessary. In the trailing edge of motile cells, talin is cleaved by the  $\text{Ca}^{2+}$ -dependent protease, calpain II, into the globular head domain (80 kDa) and the rod-shaped tail domain (190 kDa) (Franco et al. 2004; Lee et al. 2004; Shao et al. 2006). This proteolytic cleavage event prevents talin from driving integrin-mediated re-attachment to the extracellular matrix, a process that is essential to rapid focal adhesion assembly/disassembly at the leading edge of motile cells.

### ***Vinculin***

Like talin, vinculin is conformationally activated by  $\text{PIP}_2$ . Prior to activation, the C-terminal tail of vinculin binds to the N-terminal head domain and occludes binding sites within the head domain (Bakolitsa et al. 2004; Critchley 2004). Activation of vinculin by  $\text{PIP}_2$  promotes its association with talin,  $\alpha$ -actinin, Arp2/3, paxillin and actin (Bakolitsa et al. 2004; Chandrasekar et al. 2005; Critchley 2004; DeMali et al. 2002). Interestingly, vinculin is not required for focal adhesion formation as vinculin-null cells still form focal adhesions although these adhesions are smaller in size compared to wild-type cells (Critchley 2004). Rather than promote adhesion like talin, vinculin seems to stabilize focal adhesions by cross-linking talin and actin (see appendix figure 2).

### ***Focal Adhesion Kinase***

Focal adhesions kinase (FAK) is a tyrosine kinase that is activated in response to integrin engagement with the extracellular matrix. FAK plays a unique role in focal adhesion dynamics by serving as both a scaffolding protein for focal adhesion assembly and functions as a tyrosine kinase to induce focal adhesion turnover (Cox et al. 2006; Mitra et al. 2005; Mitra and Schlaepfer 2006). Structurally, FAK contains an N-terminal FERM (protein 4.1, ezrin, radixin and moesin) domain, a central catalytic domain, three proline-rich domains and a C-terminal FAT (focal adhesion targeting) domain (Cox et al. 2006; Mitra et al. 2005; Mitra and Schlaepfer 2006). The FERM, proline-rich and FAT domains all are important in FAK association with integrin, paxillin, vinculin, talin and other proteins important in focal adhesion assembly and cell motility (Cox et al. 2006; Mitra et al. 2005; Mitra and Schlaepfer 2006).

Upon integrin engagement, FAK dimerizes and autophosphorylates at Tyr<sup>397</sup>. This autophosphorylation event creates an SH2 binding domain that recruits src. Src then phosphorylates FAK on additional Tyr sites which include Tyr<sup>576/577</sup> (promotes full activation of FAK), Tyr<sup>861</sup> (SH3 domain for p130CAS association) and Tyr<sup>925</sup> (SH2 domain for paxillin, talin and p190RhoGEF) (Mitra et al. 2005). FAK also recruits and activates PLC $\gamma$ , PI3-K and p120RasGAP (Mitra et al. 2005; Mitra and Schlaepfer 2006), implicating FAK in multiple signaling pathways. Tyrosine phosphatases SHP-2 and SOCS deactivate FAK signaling and promote focal adhesion turnover (Cox et al. 2006; Mitra and Schlaepfer 2006). FAK also appears to be important in focal adhesion dynamics and cell

motility in that FAK-null fibroblasts form focal adhesions containing talin and vinculin, but these cells fail to migrate (Ilic et al. 1995).

### ***Paxillin***

Paxillin is 68 kDa scaffolding protein that contains numerous binding sites for incorporation into focal adhesions. Paxillin contains an integrin-association domain, SH2 and SH3 domains, and LIM domains which are all necessary for association with integrin, FAK, vinculin, talin and a variety of other focal adhesion proteins (Schaller 2001; Turner 2000). Paxillin is phosphorylated by the FAK/src complex on Tyr<sup>31</sup> and Tyr<sup>118</sup> to form a SH3 domain critical for Crk association (an event important in focal adhesion assembly) (Bellis et al. 1995; Schaller and Parsons 1995; Webb et al. 2005). Regulation of paxillin by Ser/Thr phosphorylation is not well understood, although Ser/Thr phosphorylation does occur prior to mitosis and upon integrin engagement with the extracellular matrix (Schaller 2001; Turner 2000; Webb et al. 2005). Like FAK, paxillin seems to be essential for focal adhesion dynamics as paxillin-null fibroblasts exhibit strong defects in cell motility and focal adhesion turnover (Hagel et al. 2002; Webb et al. 2004).

### ***SHP-2***

The SH2-domain-containing tyrosine phosphatase, SHP-2 (also known as PTP1D and PTPN11), is a non-receptor phosphatase involved in both cell migration and ERK signaling (Poole and Jones 2005). During cell migration, SHP-2 translocates from the ER to focal adhesions in response to src signaling (Wang et al. 2006) to dephosphorylate FAK

on Y<sup>925</sup> and paxillin on Y<sup>31</sup>, thus promoting focal adhesion turnover and detachment from the extracellular matrix (Vadlamudi et al. 2002). Furthermore, inhibition of SHP-2, expression of dominant-negative SHP-2 or SHP-2 knockout increase focal adhesion size and block cell motility (Inagaki et al. 2000; MacGillivray et al. 2003; Manes et al. 1999; Wang et al. 2005; Yu et al. 1998). These results suggest that SHP-2 is essential for focal adhesion dynamics in motile cells.

SHP-2 is activated by either src signaling (Tyr<sup>453</sup>) or Gab1, in which binding induces a conformational change and a subsequent elevation in phosphatase activity (Cunnick et al. 2001; Poole and Jones 2005). Studies detailing Ser/Thr regulation of SHP-2 are limited but suggest that Ser/Thr phosphorylation may play a role in modulating tyrosine phosphatase activity.

### ***Gab1***

The Grb2 associated binder 1 (Gab1) protein is a 110 kDa adapter protein that has been best characterized by its ability to bind Grb2 to modulate Ras activation (Holgado-Madruga et al. 1996; Ingham et al. 2001; Takahashi-Tezuka et al. 1998; Weidner et al. 1996). Gab1 also is recruited to the plasma membrane in response to receptor tyrosine kinase (RTK) signaling where it is phosphorylated on numerous Tyr residues to form SH2 and SH3 binding domains (Cunnick et al. 2001; Gual et al. 2000; Lehr et al. 1999; Rocchi et al. 1998). In particular, Gab1 binds to and activates PLC $\gamma$ , PI3-K and SHP-2 by inducing conformational changes in each enzyme (Gual et al. 2000). More recently, Gab1 has been

shown to mediate vascular endothelial cell migration and capillary tube formation through PI3-K and SHP-2 signaling (Rocchi et al. 1998; Yu et al. 2001).

### ***c-Abl***

Like CaMK-II, c-abl is a ubiquitously expressed, highly conserved kinase that is involved in numerous cellular functions such as cell proliferation, differentiation and cell adhesion (Hernandez et al. 2004; Plattner et al. 2003; Plattner and Pendergast 2003). c-abl, also known as Abl1, is localized to both the nucleus and cytosol. Within the nucleus, c-abl is maintained in an inactive state through direct interaction with Rb (retinoblastoma) (Hernandez et al. 2004). Upon Rb hyperphosphorylation, c-abl is activated and promotes S-phase progression by phosphorylating Tyr residues on the C-terminus of RNA Polymerase II (Hernandez et al. 2004; Plattner et al. 2003). In the cytosol, c-abl remains inactive through its interaction with PIP<sub>2</sub>. Upon signaling events (including integrin engagement with FN), PIP<sub>2</sub> is catalyzed and c-abl is activated by the src-family kinases (Plattner et al. 2003; Plattner and Pendergast 2003). c-abl can also be phosphorylated on Ser/Thr residues within its 14.3.3 protein binding domain which may influence both localization and activation.

c-abl influences cell motility by phosphorylating both paxillin and Crk to promote attachment to extracellular matrix through integrin receptors (Plattner et al. 2003). Likewise, c-abl is involved in actin cytoskeleton rearrangements through both direct interaction with F-actin and activation of small GTPases, Rho and Rac (Plattner et al. 2003).

## Rationale

Cell motility is a complex cellular process that is essential throughout embryonic development, cancer cell metastasis, and immune surveillance. An important component to cell motility is the cyclical formation and breakdown of focal adhesions (known as focal adhesion turnover). Focal adhesion formation involves the attachment of integrins to components of the extracellular matrix and the subsequent recruitment of focal adhesion-associated proteins such as FAK (focal adhesion kinase), paxillin, vinculin, talin, et al. While many studies have focused on the formation of focal adhesions in motile cells, much less is understood regarding the requisite breakdown of focal adhesions.

Previous studies have implicated CaMK-II in cell motility in various cell types. For example, in Chinese hamster ovary (CHO) cells, a mutation of Thr<sup>38</sup> to Ala<sup>38</sup> on ICAP-1 $\alpha$  (integrin cytosolic domain associated protein 1  $\alpha$ ) prevents CaMK-II phosphorylation and initiates cell spreading in a similar fashion to CaMK-II inhibition. In human mammary epithelial (HME) cells, CaMK-II has been shown to phosphorylate  $\beta$ 1 integrin on Thr<sup>788</sup> and Thr<sup>789</sup>. While both of these studies have clearly suggested a role for CaMK-II phosphorylation in motility, neither study clearly defines the notion that CaMK-II activity promotes disassembly and detachment. In this study, we show that CaMK-II promotes focal adhesion disassembly by inducing tyrosine dephosphorylation of FAK and paxillin, thus enabling cell motility.

## **CHAPTER 1: Integrin Engagement with the Extracellular Matrix Activates CaMK-II**

Integrins mediate cell adhesion by linking components of the extracellular matrix with the internal cytoskeleton but also initiate signals for a variety of other cellular functions (Brakebusch and Fassler 2005). Integrins are heterodimeric transmembrane receptors that consist of both an  $\alpha$  and  $\beta$  subunit, of which 24 different combinations are known (Hynes 2002). In embryonic fibroblasts, the primary integrin heterodimer is  $\alpha 5 \beta 1$ , which binds to fibronectin (FN) (Brakebusch and Fassler 2005; Hynes 2002). Previous studies have shown that integrin engagement with FN induces localized  $\text{Ca}^{2+}$  transients that are critical for both focal adhesion turnover and cell motility (Conklin et al. 2005; Franco et al. 2004; Giannone et al. 2004; Giannone et al. 2002; Lee et al. 2005; Marks and Maxfield 1990; Shao et al. 2006).

In differentiating P19 neurons, the principal integrin complex is  $\alpha 6 \beta 1$ , which binds laminin-1 (EHS laminin) (Brakebusch and Fassler 2005). Laminin is an extracellular matrix protein that influences preneuronal cell migration and epithelial morphogenesis and promotes neurite outgrowth and axonal specification (Ekblom et al. 2003; Miner and Yurchenco 2004). Similar to FN, integrin engagement with laminin increases cytosolic  $\text{Ca}^{2+}$  levels, an event that can cause profound effects on developing neurons. Specifically, transient elevations in  $\text{Ca}^{2+}$  influence gene expression, neurite morphogenesis, and axon guidance (Bolsover 2005; Easley et al. 2006; Henley and Poo 2004; Henley et al. 2004).



While integrin receptor activation has been shown in several cell types to induce localized  $\text{Ca}^{2+}$  transients, the targets of these transients have not been fully examined. In both neurons and fibroblast cells, CaMK-II, a  $\text{Ca}^{2+}$ /calmodulin dependent kinase, has been implicated as a potential target of integrin-mediated  $\text{Ca}^{2+}$  release and has been shown to be important in cell motility and neurite outgrowth (Blystone et al. 1999; Easley et al. 2006; Illario et al. 2005). However, elevations in CaMK-II activity have not been specifically evaluated in response to integrin engagement with components of the extracellular matrix. In this study, we show that CaMK-II is activated by integrin receptor engagement with either FN or laminin in two different cell lines, NIH/3T3 fibroblasts and P19 neurons.

## Materials and Methods

*NIH/3T3 culture-* NIH/3T3 cells were cultured on tissue culture dishes (Nunc, Rochester NY) at 37°C in DMEM with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad CA). Cells were sub-cultured every 3 to 4 days, never exceeding 95% confluency. When specified, dishes were pre-incubated with 1 µg/ml human fibronectin (Invitrogen) in PBS (Phosphate Buffered Saline) for 1 hour at 37°C or overnight at 4°C, washed once with PBS and then placed into DMEM/10% FBS just prior to plating cells.

*P19 embryonal carcinoma cell culture-* The P19 mouse diploid cell line was derived from an embryonic day 7 (E7) embryo and can be induced to differentiate by retinoic acid via embryoid body formation (McBurney et al. 1982). Undifferentiated P19 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS). For the induction of differentiation, cells were cultured at  $1 \times 10^5$  cells/ml in DMEM, 5% FBS,  $5 \times 10^{-7}$  M all trans-retinoic acid (ATRA) in bacteriological Petri dishes for 4 days (Yao et al. 1995). Induction yields embryoid bodies, which were then plated at  $2-5 \times 10^5$  cells/cm<sup>2</sup> on culture dishes. Dishes were precoated with EHS laminin (Invitrogen) in neurobasal medium containing N2 supplement (Invitrogen). Poly-L-lysine (Sigma Chemical, St. Louis, MO) pretreatment was at 0.01% for 1 hour in dH<sub>2</sub>O. The only culture modification was that cytosine arabinoside, which suppresses the growth of undifferentiated cells, was not added after plating (Yao et al. 1995).

*Whole cell lysate preparation-* Cells were harvested with trypsin–EDTA and then washed with ice-cold PBS. Pellets were immediately resuspended in ice-cold homogenization buffer, which consisted of 30 mM HEPES, pH 7.4, 2.6 mM EGTA, 20 mM MgCl<sub>2</sub>, 80 mM β-glycerol phosphate, 0.1 μM okadaic acid (Life Technologies), 0.01 mg/ml each chymostatin, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor (Sigma). Samples were then sonicated (two 5-second bursts on ice), centrifuged at 10,000×g for 15 minutes at 4°C. This buffer was previously optimized for maximal CaMK-II recovery (Tombes et al. 1995; Tombes et al. 1999). Protein concentrations were determined using the BCA assay (PIERCE, Rockford, IL).

*CaMK-II activity assay-* Total CaMK-II activity was assessed by measuring phosphate incorporation into a peptide substrate. Reactions were carried out in a total volume of 25 μl containing final concentrations of 20 mM HEPES (pH 7.4), 0.1 mM dithiothreitol, 15 mM magnesium acetate, 20mM β-glycerophosphate, 0.5 μM PKA inhibitor peptide, 0.1 μM okadaic acid, 40 μM sodium orthovanadate, 0.5 mCi [γ-<sup>32</sup>P-ATP], 35 μM autocamtide-2 (peptide substrate), 1 μM calmodulin, 1 mM EGTA, and 3 mM Ca<sup>2+</sup>. After 10 minutes at 30°C, 20 μl was pipetted onto P81 phosphocellulose paper squares that were air dried for 1 minute and washed five times in 500 ml 1% phosphoric acid. Dried paper squares were quantitated by Cerenkhov counting. The sequence of autocamtide-2 is KKALRRQETVDAL. These assay conditions were optimized for compatibility with the buffer in which cell lysates were prepared (Tombes et al. 1995; Tombes et al. 1999). %

CaMK-II activation is determined as the percentage of total CaMK-II activity that is  $\text{Ca}^{2+}$  independent.

*Immunolocalization-* Cells were fixed in methanol at  $-20^{\circ}\text{C}$  for 5 minutes for integrin staining. All other samples were fixed in 4% formaldehyde, phosphate-buffered saline (PBS), at  $4^{\circ}\text{C}$  for 15 minutes. Formaldehyde-fixed cells were then permeabilized for 5 minutes in 0.1% NP40, PBS. All fixed cells were blocked for 30 minutes in Tris-buffered saline, pH 7.4, with 0.1% Tween 20 (TBST), containing 5% bovine serum albumin and 2% appropriate preimmune serum. Cells were then incubated in primary antibody at 2–5  $\mu\text{g}/\text{ml}$  for 2 hours in 2% BSA, TBST, washed three times in TBST, incubated in secondary antibody at 2  $\mu\text{g}/\text{ml}$  in blocking solution for 1 hour, washed three times in TBST, and imaged or stored in PBS at  $4^{\circ}\text{C}$  until ready for imaging.

*Reagents-* The CaMK-II phosphor-Thr<sup>287</sup> antibody was from Upstate Biotechnology Inc (Lake Placid, NY). The  $\beta 1$  integrin blocking antibody was from BD Biosciences (San Jose, CA). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The RGD peptide (GRGDNP) is a competitive inhibitor of fibronectin binding to the  $\beta 1$  subunit of integrin and was obtained from GenScript Corp (Scotch Plains NJ). An alternative non-competing peptide (GRADSP) was obtained from Calbiochem. These peptides were incubated with cells just prior to plating at final peptide concentrations of 1 mM. KN-93, a reversible calmodulin antagonist of CaMK-I, CaMK-II, and CaMK-IV, and KN-92, an inactive analog (Sumi et al. 1991) were obtained from

CalBiochem(La Jolla, CA). The sequence of the myristoylated auto-inhibitory peptide (myr-AIP) was KKALRRQEAVDAL (BioMol, Plymouth Meeting PA). The stock concentration of these compounds was 5mM in dH<sub>2</sub>O.

*Statistical Analyses-* For CaMK-II activity assays, the average value +/- standard error are shown from samples performed in triplicate. Data are representative of multiple experiments. Statistical significance was determined with Student's t-tests with  $p < 0.05$ .

## Results

***β1 integrin engagement with fibronectin activates CaMK-II in NIH/3T3 cells.*** FN binding to β1 integrin has been shown to induce localized transient  $\text{Ca}^{2+}$  elevations (Praetorius et al. 2004). To determine whether CaMK-II is a target of these  $\text{Ca}^{2+}$  transients, the degree of CaMK-II activation was measured in NIH/3T3 cells 3 hours after sub-culturing on dishes pre-coated with 1 μg/ml FN. To avoid the influence of other adhesion molecules such as vitronectin, cells were cultured in serum-free medium. Fibroblasts sub-cultured on 1 μg/ml laminin did not spread (Fig 1A) and exhibited a significant reduction ( $p < 0.01$ ) in CaMK-II activation as compared to cells cultured on FN (Fig. 1B). Pre-incubation with either an anti-β1 integrin blocking antibody or a peptide containing Arginine-Glycine-Aspartate (RGD) decreased cell spreading on FN and significantly reduced ( $p < 0.01$ ) CaMK-II activation as compared to incubation with a control IgG or a control peptide (RAD).

***β1 blockade prevents natural CaMK-II activation in P19 neurons.*** Integrin activation by laminin in neurons has also been shown to induce localized  $\text{Ca}^{2+}$  transients (Bixby et al. 1994; Gomez et al. 2001; Kuhn et al. 1998). To demonstrate that CaMK-II is activated in response to integrin engagement with laminin, neurons were grown on laminin for 22 hours, treated for 2 hours with the indicated CaMK-II inhibitor or integrin blocking antibody (CD29), harvested and quantified for CaMK-II activity. Although assays of cell

lysates are reflective of the entire culture (neuronal and non-neuronal cells), activated CaMK-II is predominantly neuronal. CaMK-II activation in untreated P19 neurons was higher (7.3%) than in uninduced P19 embryonal carcinoma cells (3.2%), a result consistent with the natural activation of CaMK-II during neuronal induction (Figure 2). Likewise, disruption of  $\beta 1$  integrin engagement with laminin reduced CaMK-II activity similar to treatment with CaMK-II inhibition (Figure 2).

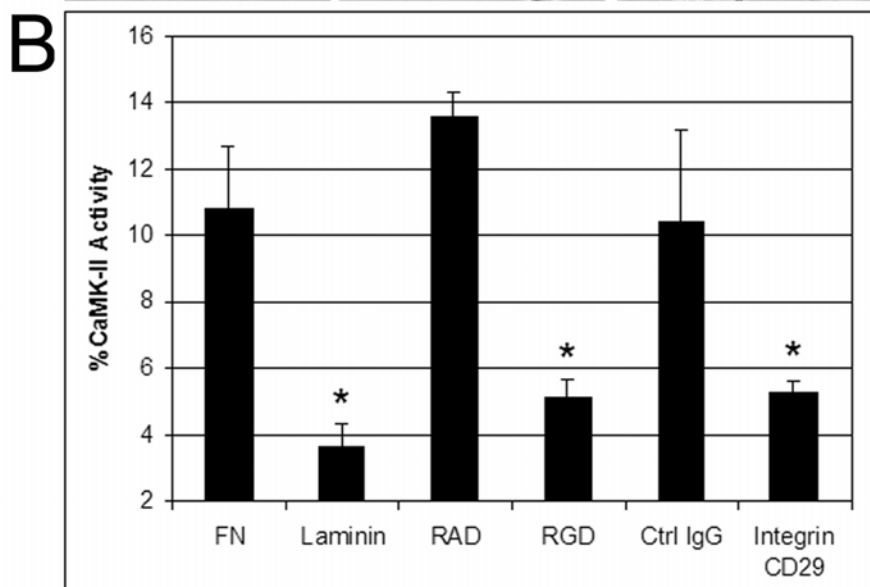
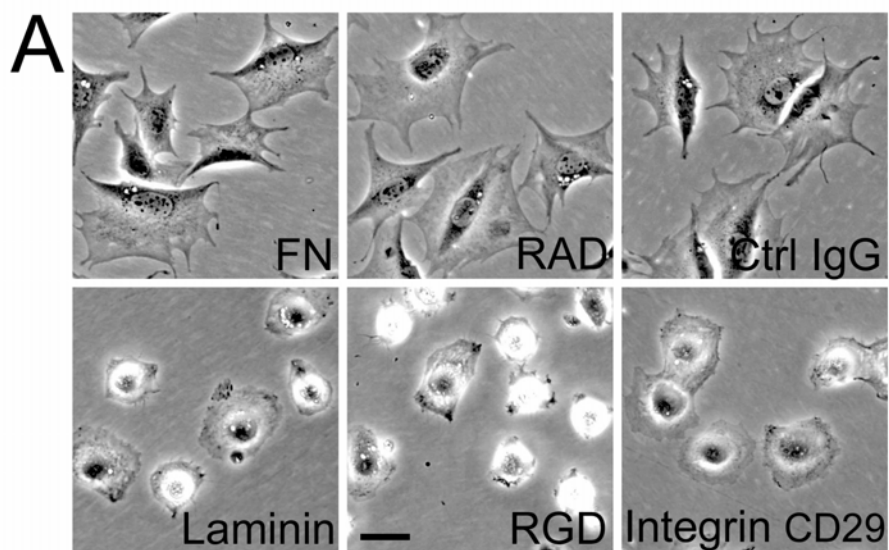
Because both protein expression and basal levels of CaMK-II activity increase upon neural induction (Johnson et al. 2000), P19 neurons can be stained with a phospho-specific CaMK-II antibody directed at the autophosphorylation site, Thr<sup>287</sup>. To complement CaMK-II activity data, P19 neurons were cultured on laminin for 22 hours and then left untreated or treated with CaMK-II inhibitors (as indicated) or CD29 for 2 hours (Figure 3). Neurons were then fixed and stained for phospho-Thr<sup>287</sup>. Neurons treated with either CaMK-II inhibitors (KN-93 or myr-AIP) or CD29 exhibited shortened neurites and a decrease in activated CaMK-II (Figure 3). Taken together, these results indicate that CaMK-II is activated in response to  $\beta 1$  integrin engagement with either FN (in fibroblast cells) or laminin (in P19 neurons).

**Figure 1. Disruption of the FN/ $\beta$ 1 interaction reduces CaMK-II Activation. (A)**

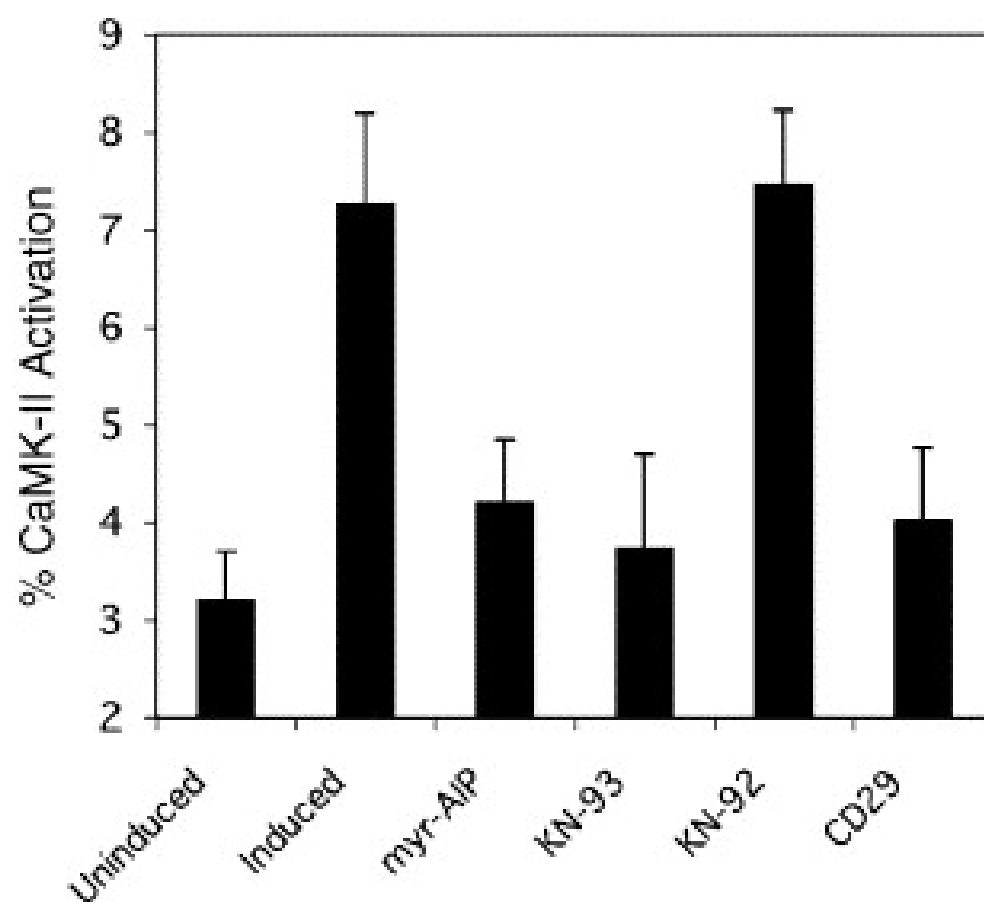
Effects of integrin  $\beta$ 1 blocking on cell attachment. NIH/3T3 cells were sub-cultured on 1  $\mu$ g/ml FN or on 1  $\mu$ g/ml laminin for 3 hours in the presence of the indicated compound.

Scale bar, 20  $\mu$ m. (B) Integrin  $\beta$ 1 blocking reduces CaMK-II activity. % CaMK-II activation was determined in cell lysates as the percentage of total (+Ca<sup>2+</sup>) activity that is Ca<sup>2+</sup> independent (-Ca<sup>2+</sup>). Samples tested represent mean values  $\pm$  SD from 3 separate experiments performed in triplicate.

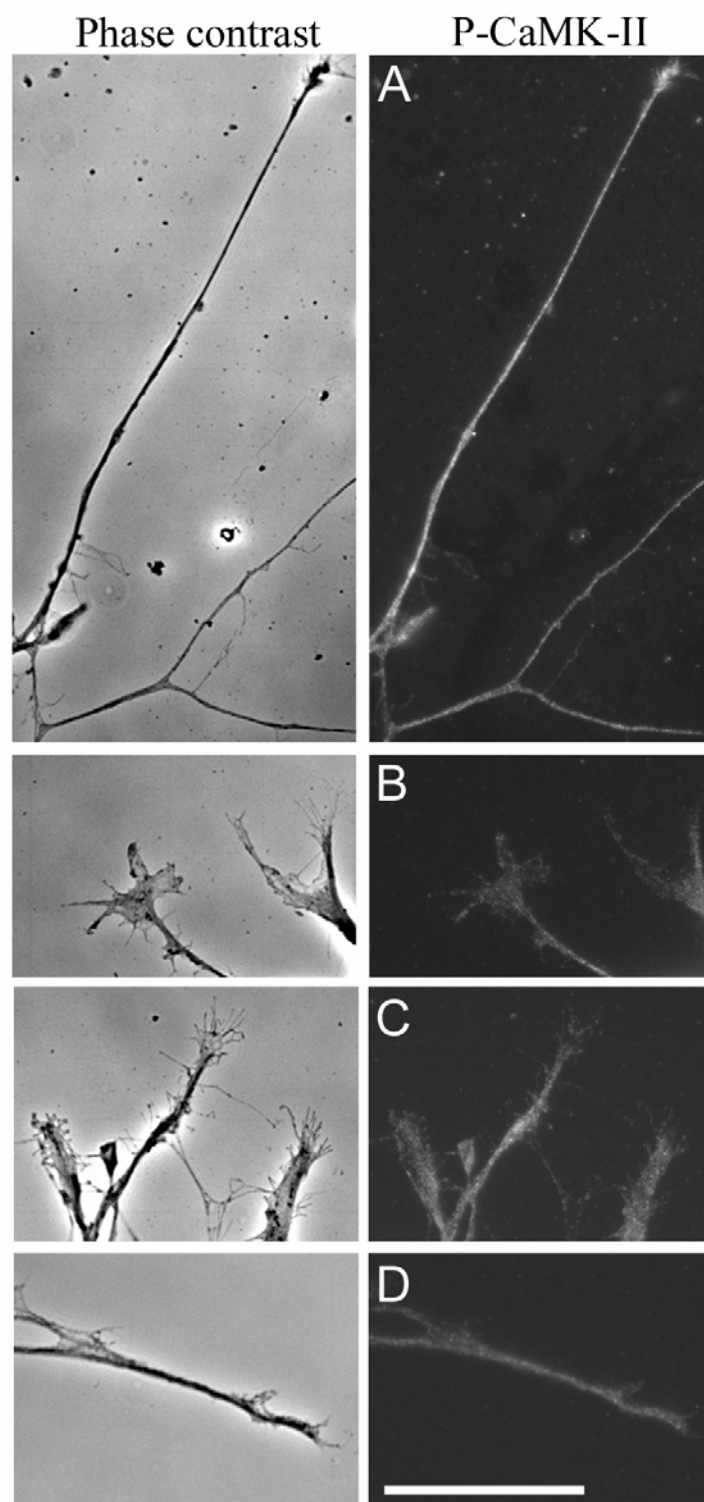




**Figure 2. Anti- $\beta$ 1 integrin inhibits CaMK-II activation.** Embryoid bodies were cultured on laminin for 22 hours, treated for 2 hours and then harvested. CaMK-II enzymatic activity was measured in cell lysates of uninduced (non-retinoic acid treated) P19 embryonal carcinoma cells and of induced (retinoic acid treated) P19 neurons after treatment with 5  $\mu$ M myr-AIP, 5  $\mu$ M KN-93, 5  $\mu$ M KN-92, and 10  $\mu$ g/ml of the CD29 anti- $\beta$ 1 integrin antibody. Percent CaMK-II activation represents  $\text{Ca}^{2+}$  independent activity as a percentage of  $\text{Ca}^{2+}$ -dependent activity. Samples tested represent mean values  $\pm$  SD from 3 separate experiments performed in triplicate.



**Figure 3. Anti- $\beta$ 1 integrin reduces active CaMK-II in collapsed axons.** 22 hours after plating on laminin, P19 neurons were left untreated (A) or treated with 5  $\mu$ M KN-93 (B), 5  $\mu$ M myr-AIP (C) or 10  $\mu$ g/ml CD29 (D) for 2 hours. Cultures were then fixed and stained with the anti-phospho-Thr<sup>287</sup> antibody (phospho-CaMK-II) and representative images from 3 separate experiments were processed identically. Scale bar represents 50  $\mu$ m.



## **CHAPTER 2: CaMK-II Promotes Focal Adhesion Disassembly**

Cell migration occurs during embryonic development, wound healing, immune surveillance and cancer metastasis and involves dozens of molecular players (Lauffenburger and Horwitz 1996; Ridley et al. 2003). Essential to cell migration is the formation and turnover of structures known as focal adhesions. Focal adhesions are critical signaling and structural hubs found in migrating cells (Huttenlocher et al. 1995; Lauffenburger and Horwitz 1996; Ridley et al. 2003) which assemble in response to the interaction of extracellular matrix ligands with integrin receptors (Huttenlocher et al. 1995; Lauffenburger and Horwitz 1996; Ridley et al. 2003).

Integrin engagement with the extracellular matrix stimulates the tyrosine phosphorylation of key cytoskeletal proteins, beginning with FAK autophosphorylation on Y<sup>397</sup> (Mitra et al. 2005). FAK autophosphorylation recruits src to focal adhesions, and the FAK/src complex phosphorylates two adapter proteins, paxillin and p130CAS (Bellis et al. 1995; Cary et al. 1998; Mitra et al. 2005; Schaller and Parsons 1995; Webb et al. 2004). Phosphorylated paxillin and p130CAS recruit proteins, such as Crk, to focal adhesions through SH2/SH3 binding domains. Fibroblasts deficient in either FAK or paxillin exhibit defects in cell migration (Hagel et al. 2002; Ilic et al. 1995), suggesting that both proteins are essential for adhesion dynamics (Webb et al. 2004).

Transient elevations in  $\text{Ca}^{2+}$  have been shown to induce the disassembly of focal adhesions (Conklin et al. 2005; Giannone et al. 2004; Giannone et al. 2002; Marks and Maxfield 1990). While calcineurin and calpain have been implicated as  $\text{Ca}^{2+}$  targets which influence focal adhesion dynamics (Conklin et al. 2005; Franco et al. 2004; Lee et al. 2005; Shao et al. 2006), the type II  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (CaMK-II) has also emerged as a potential target of fibronectin (FN)-induced  $\text{Ca}^{2+}$  transients (Blystone et al. 1999; Illario et al. 2005).

CaMK-II is encoded by four genes ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) to yield over three dozen splice variants (Tombes et al. 2003), some of which interact with the actin cytoskeleton (Easley et al. 2006; Faison et al. 2002; Fink et al. 2003; Lin et al. 2004; Shen et al. 1998). CaMK-II has been shown to influence cell motility during embryogenesis and in cultured mammalian cells. Morphogens encoded by certain members of the *wnt* gene family activate CaMK-II and lead to convergent extension cell movements during and after gastrulation (Kohn and Moon 2005; Kuhl et al. 2000; Sheldahl et al. 2003). CaMK-II is also necessary for the attachment and motility of human mammary epithelial cells (HME), Chinese hamster ovary cells (CHO) and vascular smooth muscle cells (VSM) (Bilato et al. 1997; Bouvard and Block 1998; Bouvard et al. 1998; Lundberg et al. 1998; Pauly et al. 1995; Pfeleiderer et al. 2004; Suzuki and Takahashi 2003), and has been implicated in integrin cross-talk (Blystone et al. 1999). While these studies emphasize the importance of CaMK-II in cell motility, the mechanism by which CaMK-II influences motility and adhesion dynamics remains unknown.

To define the mechanism by which CaMK-II influences NIH/3T3 fibroblast cell motility, GFP-tagged wild type and constitutively active CaMK-IIs were used in conjunction with membrane permeant CaMK-II inhibitory drugs in localization, motility and focal adhesion assays. Even though a direct substrate has not been identified, the results of this study indicate that CaMK-II catalytic activity promotes focal adhesion disassembly and detachment from the extracellular matrix by inducing the tyrosine dephosphorylation of focal adhesion proteins, thus enabling cell motility.



## Materials and Methods

*NIH/3T3 culture and harvesting-* NIH/3T3 cells were cultured on tissue culture dishes (Nunc, Rochester NY) at 37°C in DMEM with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad CA). Cells were sub-cultured every 3 to 4 days, never exceeding 95% confluency. When specified, dishes were pre-incubated with 1 µg/ml human fibronectin (Invitrogen) in PBS (Phosphate Buffered Saline) for 1 hour at 37°C or overnight at 4°C, washed once with PBS and then placed into DMEM/10% FBS just prior to plating cells. Cells were harvested by trypsinization, washed in ice-cold PBS and then resuspended in homogenization buffer, which consisted of 30 mM Hepes pH 7.4, 20 mM MgCl<sub>2</sub>, 80 mM β-glycerol phosphate, 2.6 mM EGTA, 0.1 µM okadaic acid, 1 µg/ml each chymostatin, leupeptin, antipain, pepstatin and soybean trypsin inhibitor. Cells were lysed using two four-second bursts from a probe sonicator (Misonix, Farmingdale NY) and then centrifuged at 12,000 x g for 15 minutes at 4°C.

*Plasmid Constructs-* EGFP-linked CaMK-II constructs used in this study were prepared as previously described (Lantsman and Tombes 2005). EGFP-paxillin, dsRed-paxillin and EGFP-FAK were prepared as described (Brown et al. 2006; Webb et al. 2004). EGFP-talin and vinculin were generous gifts from Dr. Kenneth Yamada, National Institutes of Health, Bethesda MD and Dr. Benjamin Geiger, Weizmann Institute of Science, Rehovot Israel, respectively.

*Transfection and Microscopy*- Freshly sub-cultured cells were transfected with Lipofectamine 2000 as specified (Invitrogen). Live or formaldehyde fixed cells were imaged in phase contrast, traditional fluorescence (Fm) or Total Internal Reflection Fluorescence microscopy (TIRFm) using an IX-70 inverted microscope equipped with a 12-bit black/white F-View CCD camera and processed using Microsuite-B3SV Version 3.2 software (Olympus, Melville NY). TIRF Illumination utilized a 10mW Argon-ion laser (Melles Griot, Carlsbad CA) for 488nm illumination via a 60X/1.45NA PlanApo objective (Olympus). Living cells were maintained at 37°C using a stage heater (20/20 Technology Inc., Wilmington NC)

*Analysis of Cell Migration, Spreading and Focal Adhesion Size*- NIH/3T3 cells were cultured to confluency and then scratch-wounded using a single stroke from a fine pipette tip. Cells were immediately re-fed with fresh 10% FBS/DMEM containing either vehicle alone, 10  $\mu$ M KN-93 or 20  $\mu$ M myr-AIP. Cells were then imaged under phase contrast at 0, 3 and 6 hours post wounding. The microscope stage was maintained at 37°C. Between imaging sessions, cells were returned to the incubator. Cell migration rates were calculated from images taken 1 and 4 hours after plating on FN with Microsuite-B3SV Version 3.2 software (Olympus). Diameter measurements for cell spreading and focal adhesion size analysis were also computed with Microsuite-B3SV Version 3.2 software. Minimum and maximum threshold values for focal adhesion size were 1.7  $\mu$ m<sup>2</sup> and 35  $\mu$ m<sup>2</sup>, respectively. Student's T-tests were used for statistical analysis where significance is indicated.

*Immunoblotting-* Transfected NIH/3T3 cells were harvested as described above (see *Cell Culture and Harvesting*). Immunoblots were blocked with 5% BSA, TBSTA containing 5% goat serum. Blots were then incubated overnight with 1 µg/ml primary antibody in 5% BSA, TBSTA. After a 2 hour incubation with 2 µg/ml alkaline phosphatase conjugated secondary antibodies, blots were developed with 0.25 mg/ml BCIP/NBT (Roche, Indianapolis IN) in phosphatase buffer (Lantsman and Tombes 2005).

*Reagents-* Total FAK and paxillin antibodies were obtained from BD Biosciences (Rockville, MD), phospho-Y<sup>31</sup> paxillin and phospho-S<sup>843</sup> FAK antibodies from Biosource/Invitrogen, and phospho-Y<sup>925</sup> FAK antibody from Cell Signaling Technology (Danvers, MA). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). KN-93 (CalBiochem, La Jolla, CA) is a calmodulin antagonist, specific for a subset of CaM kinases including CaMK-II, CaMK-I and CaMK-IV (Hidaka and Ishikawa 1992; Sumi et al. 1991). However, CaMK-I and CaMK-IV were undetectable in NIH/3T3 cells as determined using immunological methods (data not shown). KN-93 inhibits CaMK-II by 50% at approximately 2 µM and by 90% at 10 µM in these cells (Tombes et al. 1995). Myristoylated autoinhibitory peptide (KKALRRQEAVDAL) is based on the CaMK-II autoinhibitory domain (Laabich and Cooper 2000). Myr-AIP inhibits purified CaMK-II by 90% at 20 µM (data not shown) and was obtained from Biomol (Plymouth Meeting, PA).

## Results

***CaMK-II Inhibition Accelerates Cell Spreading on Fibronectin.*** Fibronectin (FN) binding to  $\beta 1$  integrin has been shown to induce localized transient  $\text{Ca}^{2+}$  elevations (Praetorius et al. 2004), which activate CaMK-II (Blystone et al. 1999; Illario et al. 2005). To assess whether CaMK-II activity is necessary for cell spreading, two mechanistically distinct CaMK-II inhibitors, myr-AIP (myristoylated autoinhibitory peptide) and KN-93 were used. Myr-AIP directly blocks catalytic activity while KN-93 antagonizes CaM binding and thus prevents activation. NIH/3T3 cells were sub-cultured onto 1  $\mu\text{g}/\text{ml}$  FN in medium alone (Figure 4A), 20  $\mu\text{M}$  myr-AIP (Figure 4B), or 10  $\mu\text{M}$  KN-93 (Figure 4C) and imaged at 0, 5, 10, 15, 30, 45 and 60 minutes post plating on FN. Cells treated with either KN-93 or myr-AIP spread more rapidly than untreated cells as shown at 30, 45 and 60 minutes. KN-92, an inactive analog of KN-93, had no effect on spreading (data not shown). The degree of spreading was quantified by averaging cell diameters ( $n > 100$ ) for each condition, and treatment with either KN-93 or myr-AIP significantly accelerated spreading after 30 minutes ( $p < 0.01$ ) (Figure 4D). This effect of CaMK-II inhibition on cell spreading was dependent on FN as cells cultured on laminin, or untreated dishes, attached slowly and exhibited no sensitivity to KN-93 or myr-AIP treatment.

***CaMK-II Inhibition Reduces Cell Motility.*** Mechanical wounding of confluent cells has previously been shown to increase cytosolic  $\text{Ca}^{2+}$  levels in cells along the edge of the wound (Tran et al. 1999), which may activate CaMK-II to induce cell migration. To evaluate whether CaMK-II inhibition influences this cell motility, NIH/3T3 cells were scratch-wounded and immediately treated with fresh medium containing vehicle alone, 20  $\mu\text{M}$  myr-AIP or 10  $\mu\text{M}$  KN-93. Cells were then imaged 0, 3 and 6 hours post wounding. Cells treated with myr-AIP or KN-93 retained a sizeable wound after 6 hours while cells treated with vehicle alone exhibited >70% wound closure (Figure 5).

***CaMK-II Localizes to the Surface of Motile NIH/3T3 cells.*** Because CaMK-II inhibition profoundly affected cell spreading and motility, Total Internal Reflection Fluorescence microscopy (TIRFm) was used to examine whether CaMK-II localizes to the cell surface. NIH/3T3 cells were transfected with GFP-tagged  $\delta_{\text{C}}$  CaMK-II and then sub-cultured onto FN-coated glass coverslips. Conventional fluorescence (Fm) confirmed the perinuclear distribution of  $\delta_{\text{C}}$ , as previously reported in these cells (Caran et al. 2001; Tombes et al. 1999). TIRFm of the same cell indicated that a substantial portion of  $\delta_{\text{C}}$  CaMK-II localizes to the cell surface (Figure 6A). Time-lapse TIRFm demonstrated that CaMK-II is dynamically enriched at the leading edge of a migrating cell and in discrete patterns throughout the cell (video not included). This novel, dynamic surface localization supports a role for CaMK-II in adhesive and migratory events.

***Constitutively Active CaMK-II Causes Cell Rounding and Inhibits Cell Motility.***

CaMK-II can be made constitutively active through a phosphomimetic point mutation (T<sup>287</sup>D) in the autoinhibitory domain (Johnson et al. 2000). To test the effects of constitutively active CaMK-II on cell motility, cells were transfected with GFP-tagged constitutively active (CON)  $\delta_C$  CaMK-II, sub-cultured on FN-coated glass coverslips and imaged using Fm and TIRFm (Figure 6B). Cells transfected with CON CaMK-II exhibited a rounded morphology and lacked the discrete surface localization observed with WT CaMK-II (Figure 6). These cells also failed to migrate on FN (video not included).

***Both constitutive activation and inhibition of CaMK-II disrupt cell motility.*** To further assess the effect of CON CaMK-II expression and CaMK-II inhibition on cell migration, NIH/3T3 cells were transfected with either WT or CON  $\delta_C$  CaMK-II and sub-cultured on FN-coated coverslips. Also, untransfected cells were sub-cultured on FN in the presence or absence of CaMK-II inhibitors as indicated (Figure 7). After incubation on FN for 1 hour, cells were imaged under phase contrast to track cell migration over a 3 hour time-course (n > 15 cells for each condition). Cells expressing CON CaMK-II or treated with CaMK-II inhibitors showed a significant reduction in cell migration rates as compared to either untreated control cells or cells expressing WT CaMK-II, respectively (p < 0.01) (Figure 7).

***Constitutively Active CaMK-II Disrupts Paxillin Incorporation into Focal Adhesions.*** Because CaMK-II localizes to the cell surface and influences cell spreading

and motility, the effect of CaMK-II activity on focal adhesion proteins was next evaluated. Due to the fact that CON CaMK-II expressing cells loosely adhere to FN, GFP-linked focal adhesion proteins were used in place of endogenous immuno-localization. Thus, cells co-transfected with non-fluorescent WT or CON  $\delta_C$  CaMK-II and GFP-tagged focal adhesion proteins (paxillin, FAK, vinculin or talin) were sub-cultured on FN-coated coverslips for 3 hours and then imaged using conventional fluorescent microscopy. WT CaMK-II expressing cells exhibited robust adhesions as visualized with each of the four focal adhesion proteins (Figure 8). In contrast, cells expressing CON CaMK-II exhibited a near-complete loss of paxillin-containing focal adhesions, while FAK, vinculin and talin remained localized to focal adhesions. Paxillin was re-incorporated into focal adhesions when cells expressing CON CaMK-II were sub-cultured on FN in the presence of 20  $\mu$ M myr-AIP (CON/myr-AIP). Because paxillin was preferentially, but reversibly eliminated from focal adhesions in cells expressing CON CaMK-II, the rate of paxillin recovery to focal adhesions after myr-AIP treatment was assessed in single cells using GFP-paxillin time lapse microscopy. Adjacent non-transfected and transfected (CON CaMK-II and GFP-paxillin) cells were imaged under phase contrast and conventional fluorescent microscopy before and 60, 120 and 180 minutes after treatment with 20  $\mu$ M myr-AIP (Figure 9A). Before treatment, the transfected cell was rounded and lacked detectable paxillin-containing adhesions but exhibited GFP-paxillin fluorescence within the cytosol. Within 1 hour of myr-AIP treatment, paxillin re-incorporated into focal adhesions (Figure 9A), suggesting that CaMK-II inhibition rapidly influenced paxillin localization. To further illustrate the specific effect of CON CaMK-II on paxillin localization to focal

adhesions, NIH/3T3 cells were transfected with non-fluorescent CON  $\delta_C$  CaMK-II, vinculin-GFP and dsRed-paxillin, sub-cultured on 1  $\mu\text{g/ml}$  FN and then imaged directly before and 1 hour after treatment with 20  $\mu\text{M}$  myr-AIP. Within 1 hour treatment of myr-AIP, paxillin co-localized with vinculin in focal adhesions at the periphery of the cell (see arrowheads, Figure 9B). These results suggest that CaMK-II activity specifically affects paxillin incorporation into focal adhesions.

***Inhibition of Endogenous CaMK-II Increases the Size of Paxillin-GFP Labeled Focal Adhesions.*** The influence of endogenous CaMK-II activity on paxillin-containing focal adhesions was next evaluated. Cells were transfected with GFP-paxillin alone and sub-cultured on FN for 3 hours in the presence or absence of 20  $\mu\text{M}$  myr-AIP. Compared to untreated cells (Figure 10A), treatment with myr-AIP increased the size of GFP-paxillin adhesions (Figure 10B). Focal adhesion size was quantified for each condition ( $n > 600$  focal complexes) and confirmed this distinct shift to larger focal adhesions in cells treated with myr-AIP (Figure 10C). Furthermore, the effects of myr-AIP treatment were specific to paxillin as FAK, vinculin and talin showed no significant change in focal adhesion size in the presence of myr-AIP (Figure 10D). Thus, the inhibition of CaMK-II disrupts cell motility by stabilizing paxillin localization to focal adhesions whereas constitutively active CaMK-II blocks cell motility by destabilizing paxillin localization.

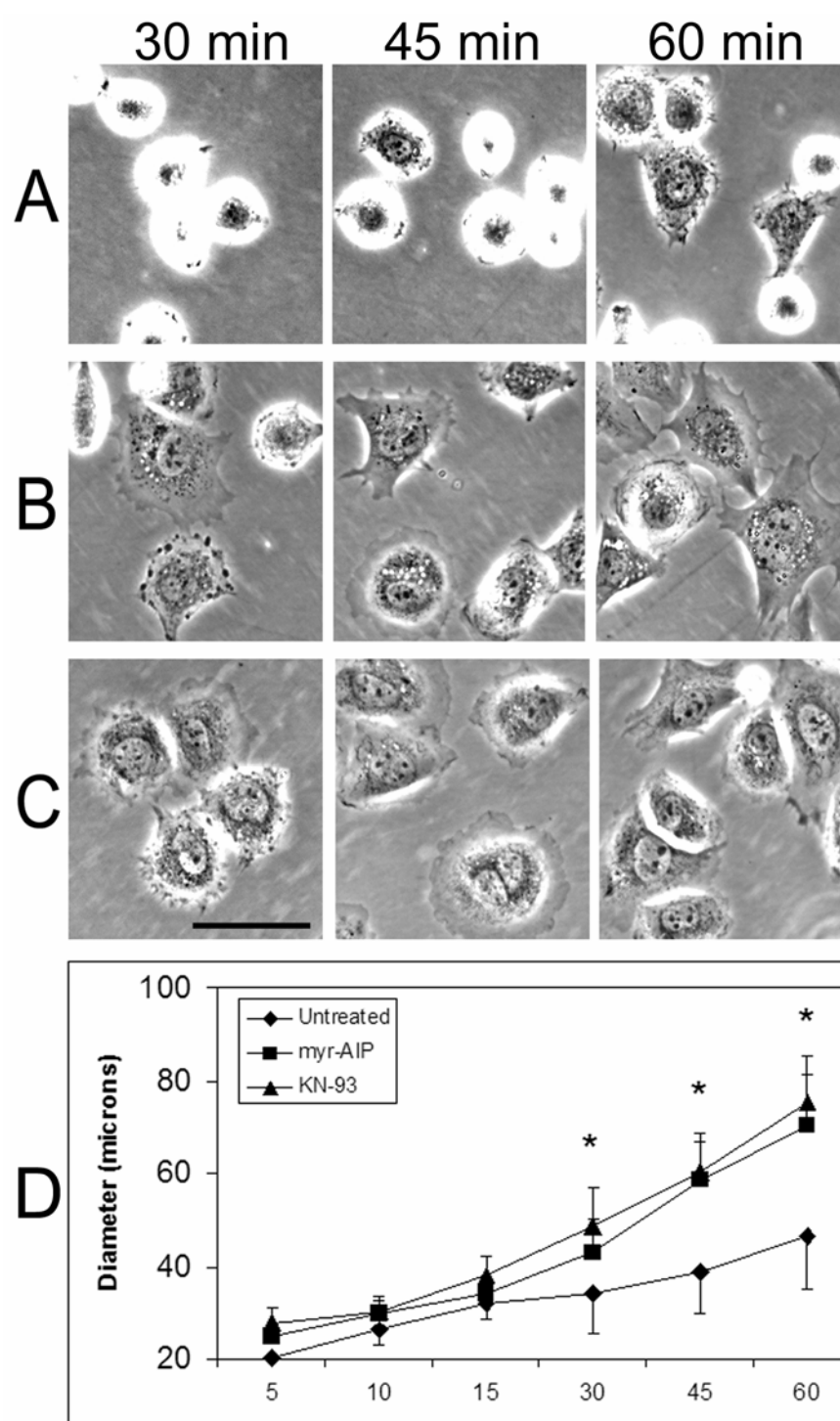
***Constitutively Active CaMK-II Decreases Phospho-tyrosine Levels on FAK and Paxillin.***  $\beta 1$  integrin-mediated cell attachment stimulates a series of tyrosine



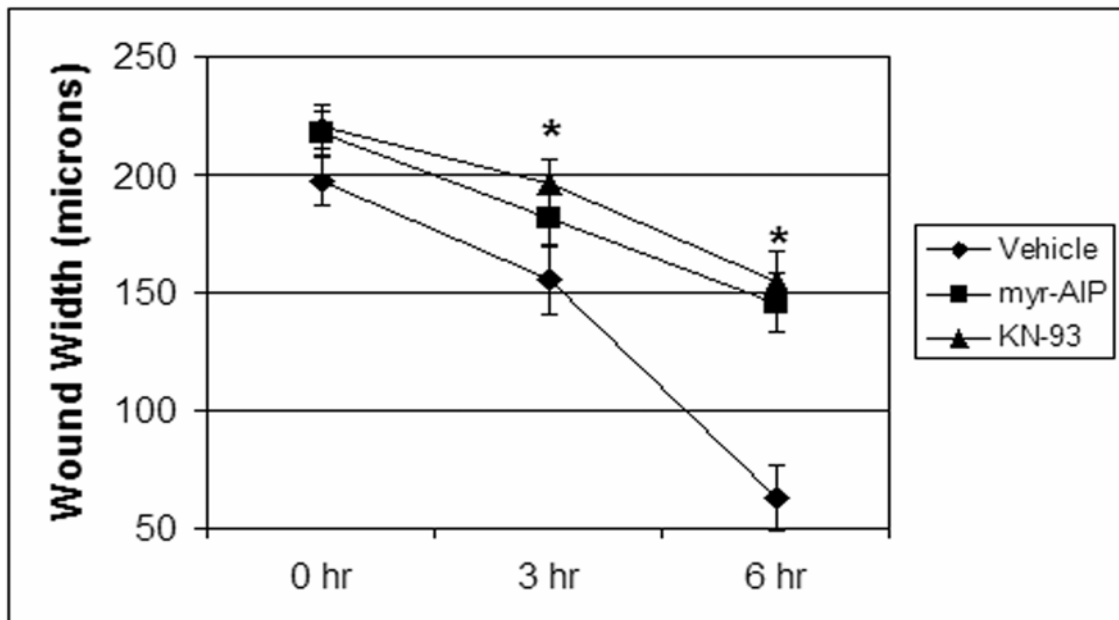
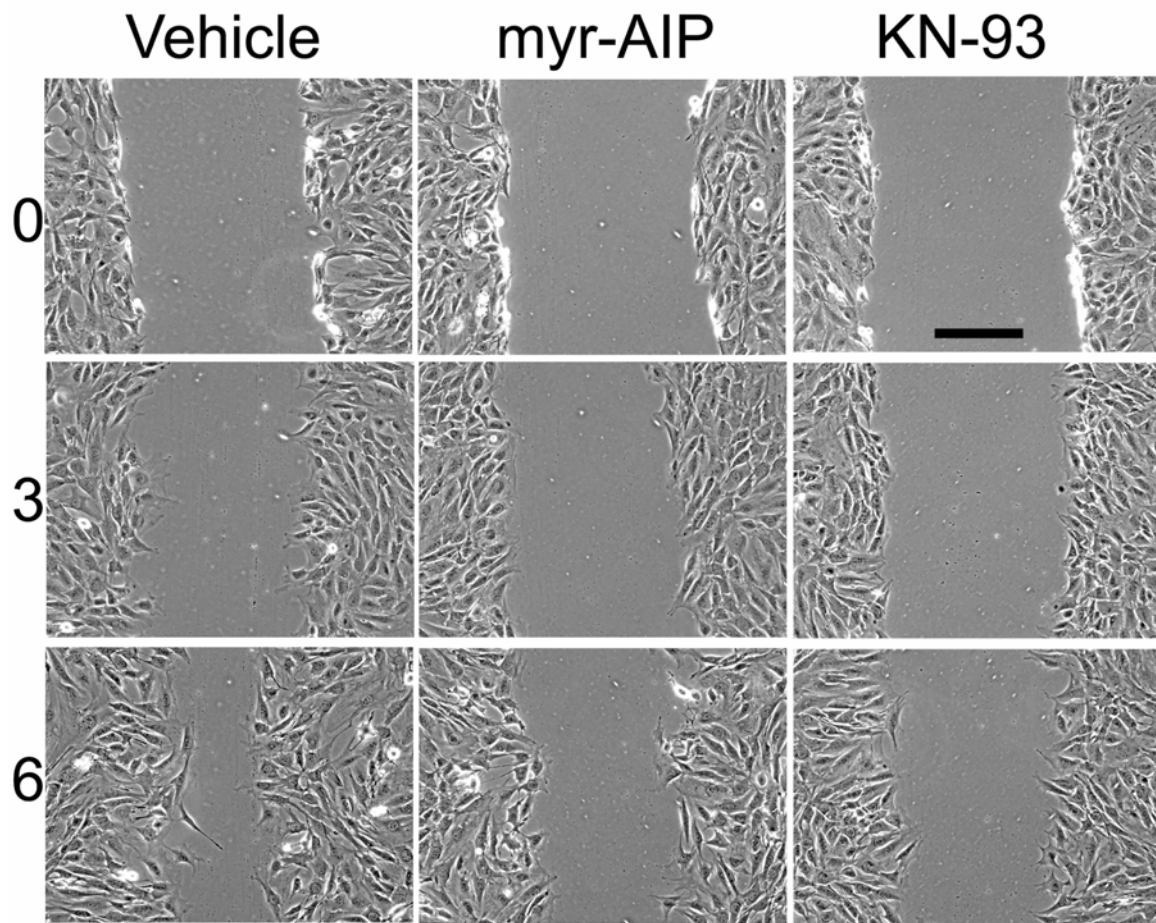
phosphorylations on adhesion proteins, which form phosphorylated SH2 and SH3 domains necessary for focal adhesion assembly (Bellis et al. 1995; Cary et al. 1998; Mitra et al. 2005; Schaller and Parsons 1995). In particular, tyrosine phosphorylation of FAK on Y<sup>925</sup> and paxillin on Y<sup>31</sup> promotes focal adhesion assembly (Bellis et al. 1995; Schlaepfer et al. 2004; Sieg et al. 2000). Because CaMK-II activity disrupted paxillin incorporation into focal adhesions, we evaluated whether CaMK-II influences tyrosine phosphorylation of FAK and paxillin by co-transfecting GFP-FAK or GFP-paxillin with either WT or CON CaMK-II. After sub-culturing transfected cells on FN for 3 hours either with or without 20  $\mu$ M myr-AIP, as described above, GFP-FAK and paxillin lysates were probed with residue-specific phospho-tyrosine antibodies (Figure 11A, B). CON CaMK-II expression significantly reduced FAK phospho-Y<sup>925</sup> relative to the level in cells transfected with WT CaMK-II, but did not impair FAK autophosphorylation (Y<sup>397</sup>). Similarly, CON CaMK-II expression significantly decreased paxillin Y<sup>31</sup> phosphorylation (Figure 11A, B). FAK Y<sup>925</sup> and paxillin Y<sup>31</sup> phospho-tyrosine levels were partially restored when CON CaMK-II expressing cells were treated with 20  $\mu$ M myr-AIP (CON/myr-AIP) (Figure 11A, B).

To determine the effects of CaMK-II activity on endogenous FAK and paxillin tyrosine levels, NIH/3T3 cells were sub-cultured on FN for three hours in the presence of either vehicle control, 10  $\mu$ M KN-93 or 20  $\mu$ M myr-AIP (Figure 11C). Inhibition of CaMK-II induced a slight increase in Y<sup>925</sup> phosphorylation on FAK and Y<sup>31</sup> on paxillin as compared to vehicle control cells without affecting total expression levels of FAK and paxillin (Figure 11C). Taken together, these results indicate that CaMK-II promotes focal adhesion disassembly by stimulating tyrosine dephosphorylation of FAK and paxillin.

**Figure 4. CaMK-II inhibition accelerates cell spreading.** NIH/3T3 cells were plated on 1  $\mu$ g/ml FN and treated with vehicle alone (A), 20  $\mu$ M myr-AIP (B) and 10  $\mu$ M KN-93 (C). Cells were live-imaged under phase contrast at 0, 5, 10, 15, 30, 45 and 60 minutes post plating as indicated. Scale bar, 50  $\mu$ m. (D) Graphical representation of diameter measurements from cells imaged at each time point for each condition.  $n > 100$  for each condition and each time point. Results obtained are representative of 3 different experiments.

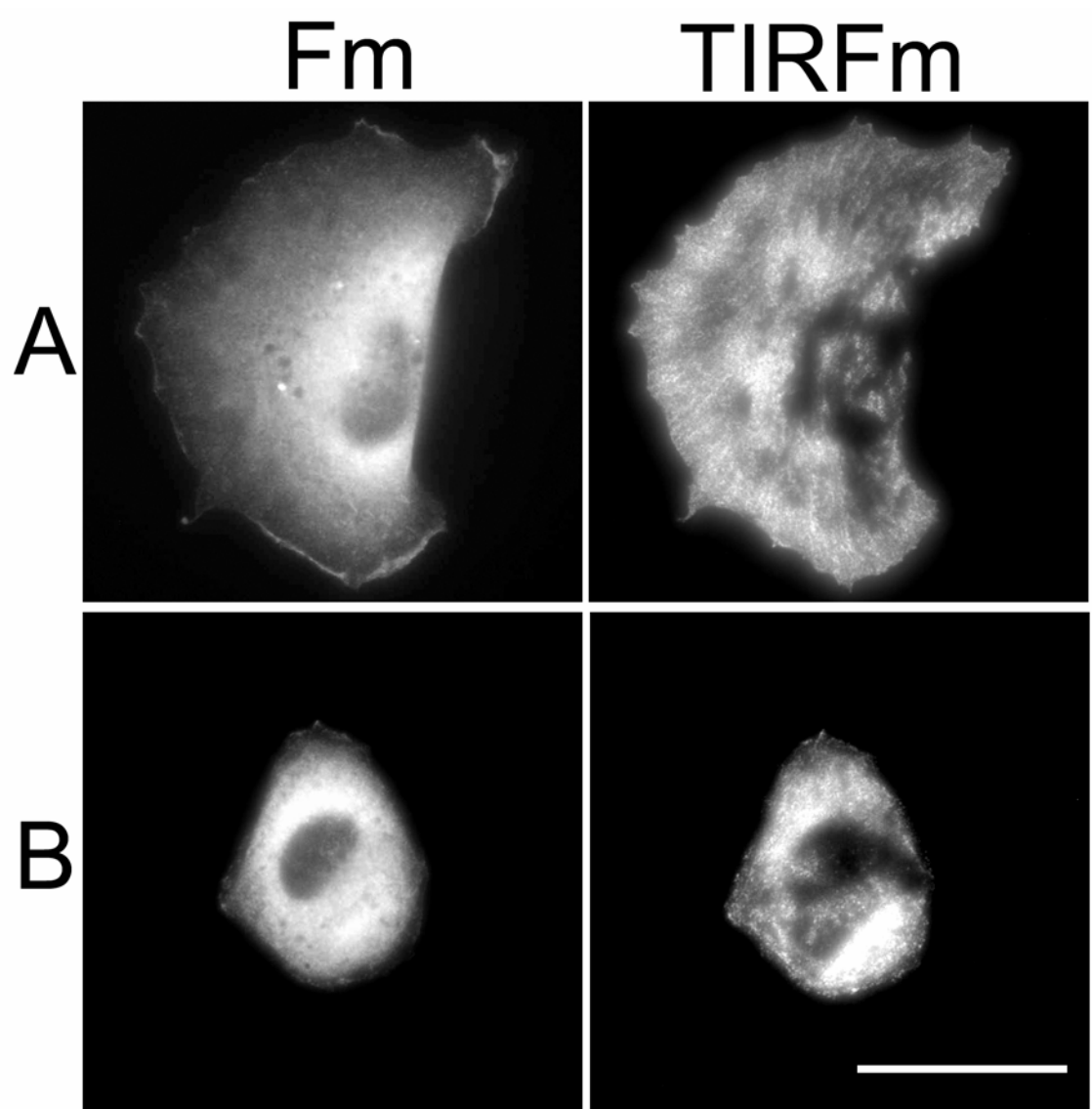


**Figure 5. CaMK-II inhibition reduces the rate of wound closure.** A confluent monolayer of cells was scratch-wounded and then imaged at 0, 3 and 6 hours in the presence of vehicle alone, 20  $\mu$ M myr-AIP or 10  $\mu$ M KN-93. Scale bar, 200  $\mu$ m. Images shown are representative from 3 separate experiments.



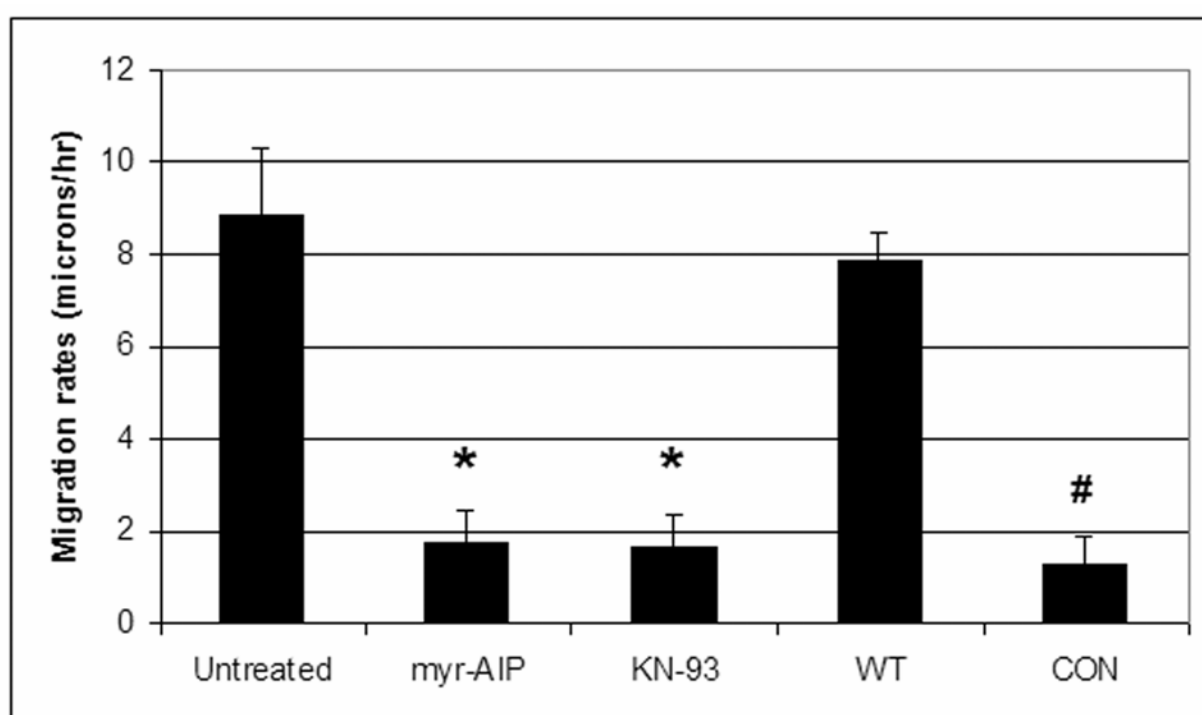
**Figure 6. CaMK-II is dynamically localized at the surface of motile fibroblasts.**

NIH/3T3 cells were transfected with either GFP-labeled wild-type  $\delta_C$  CaMK-II (A) or constitutively active (CON)  $\delta_C$  CaMK-II (B) and sub-cultured on 1  $\mu\text{g/ml}$  FN for 3 hours. Cells were fixed and imaged using traditional fluorescence microscopy (Fm) and total internal reflection fluorescence microscopy (TIRFm). Scale bar, 50  $\mu\text{m}$ .

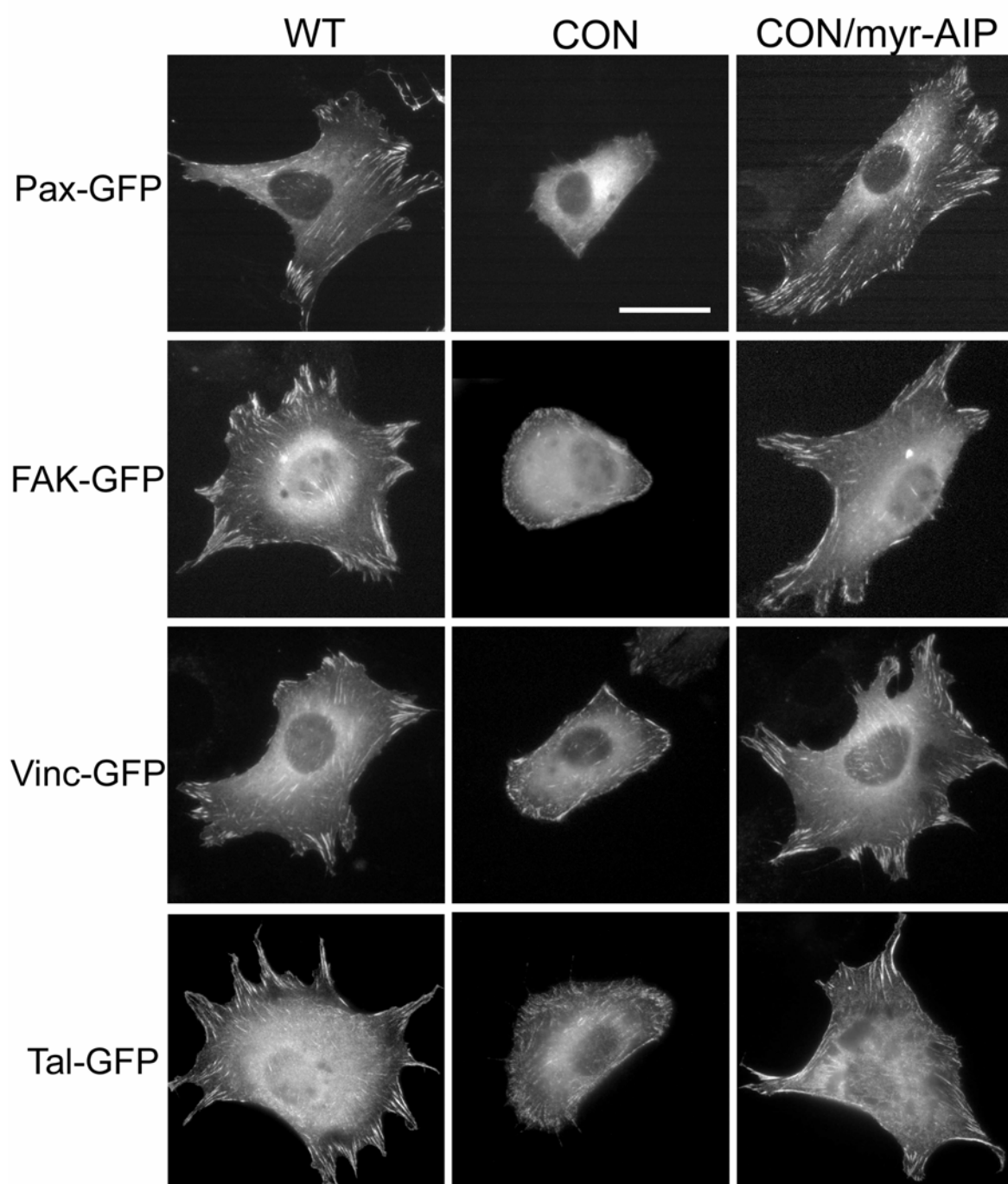


**Figure 7. Both CaMK-II inhibition and expression of constitutively active CaMK-II block cell motility.** NIH/3T3 cells were transfected with either GFP-labeled wild-type or CON  $\delta_c$  CaMK-II and sub-cultured on 1  $\mu\text{g/ml}$  FN for 1 hour. Similarly, untransfected NIH/3T3 cells were sub-cultured on FN in the presence or absence of the indicated CaMK-II inhibitor. After incubation on FN for 1 hour, cells were imaged under phase contrast over a 3 hour time-course. Migration rates (in microns/hour) were determined using Olympus Microsuite software. \*  $p < 0.01$  compared to Untreated. #  $p < 0.01$  compared to WT.

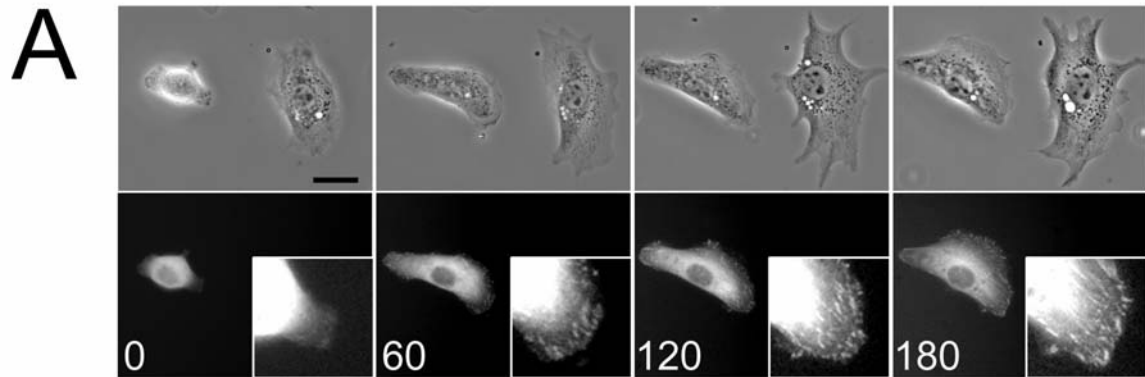




**Figure 8. Constitutively active CaMK-II impairs focal adhesion formation.** NIH/3T3 cells were co-transfected with either unlabeled wild-type or CON  $\delta_C$  CaMK-II and the indicated GFP-tagged protein. Cells were sub-cultured on 1  $\mu\text{g/ml}$  FN for 3 hours in the presence or absence of 20  $\mu\text{M}$  myr-AIP, fixed and imaged under traditional fluorescence microscopy. Scale bar, 50  $\mu\text{m}$ .

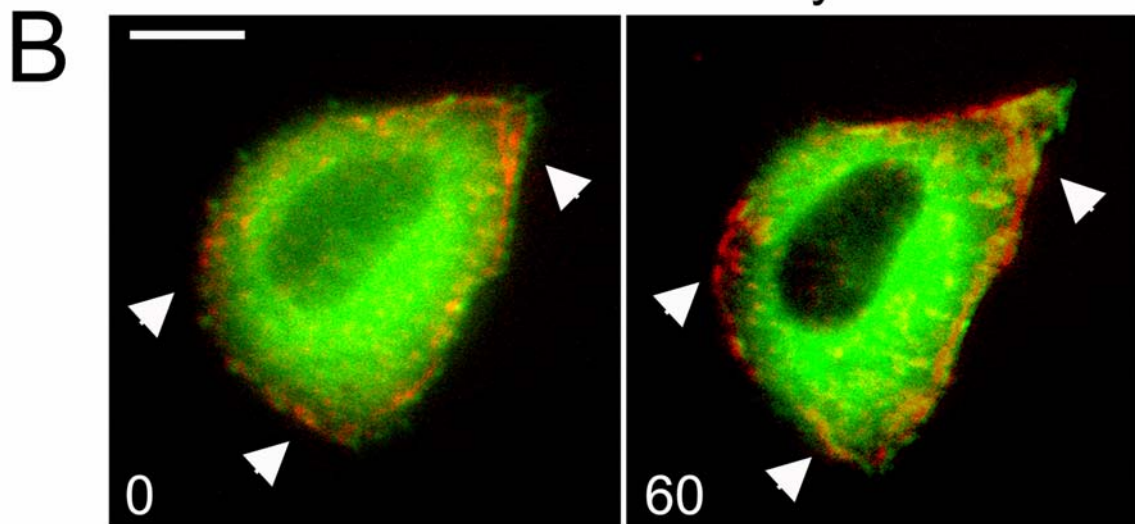


**Figure 9. Inhibition of constitutively active CaMK-II restores paxillin-containing complexes within 1 hour.** (A) NIH/3T3 cells were co-transfected with unlabeled CON  $\delta_C$  CaMK-II and GFP-paxillin. Cells were plated on 1  $\mu\text{g/ml}$  FN for 3 hours, then re-fed with medium containing 20  $\mu\text{M}$  myr-AIP. Cells were then imaged under phase contrast and traditional fluorescence microscopy at 0, 60, 120 and 180 minutes post treatment. Inset images represent the lower right-hand corner of the transfected cell. Scale bar, 50  $\mu\text{m}$ . (B) NIH/3T3 cells were transfected with unlabeled CON  $\delta_C$  CaMK-II, vinculin-GFP and dsRed-paxillin and sub-cultured on FN for 3 hours. Cells were then imaged under traditional fluorescent microscopy directly before and 1 hour after treatment with 20  $\mu\text{M}$  myr-AIP. Arrowheads indicate regions of interest where treatment of myr-AIP induces increased paxillin localization to the cell periphery and enhanced co-localization with vinculin-GFP. Scale bar, 10  $\mu\text{m}$ .



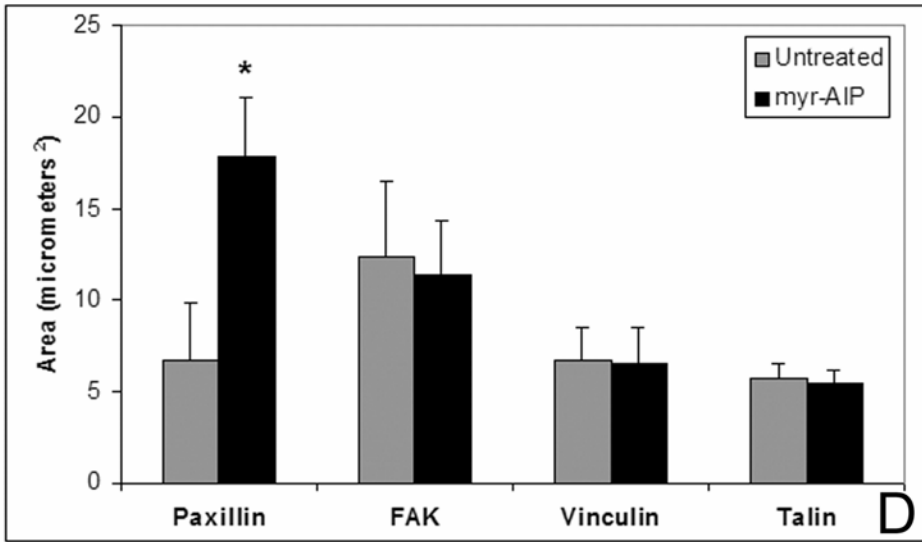
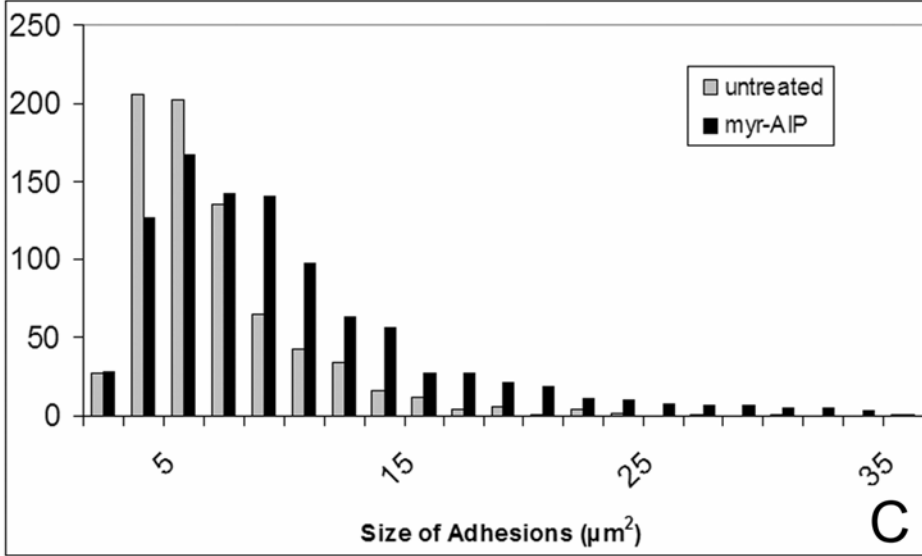
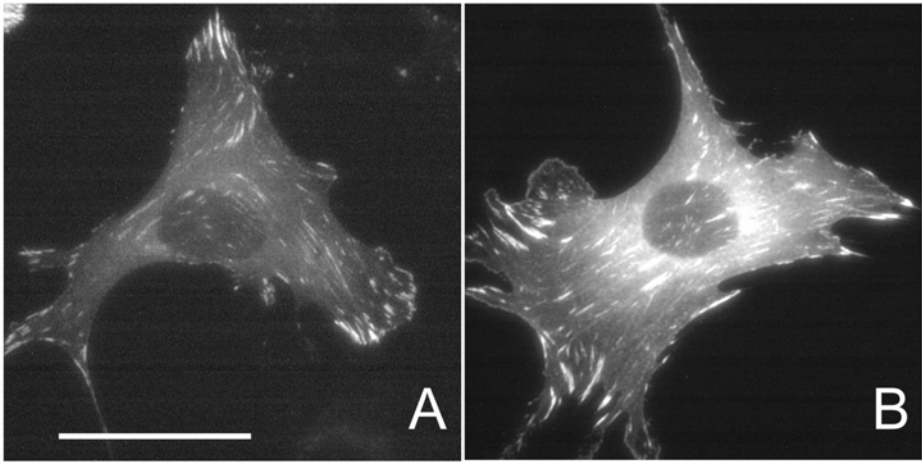
Untreated

myr-AIP



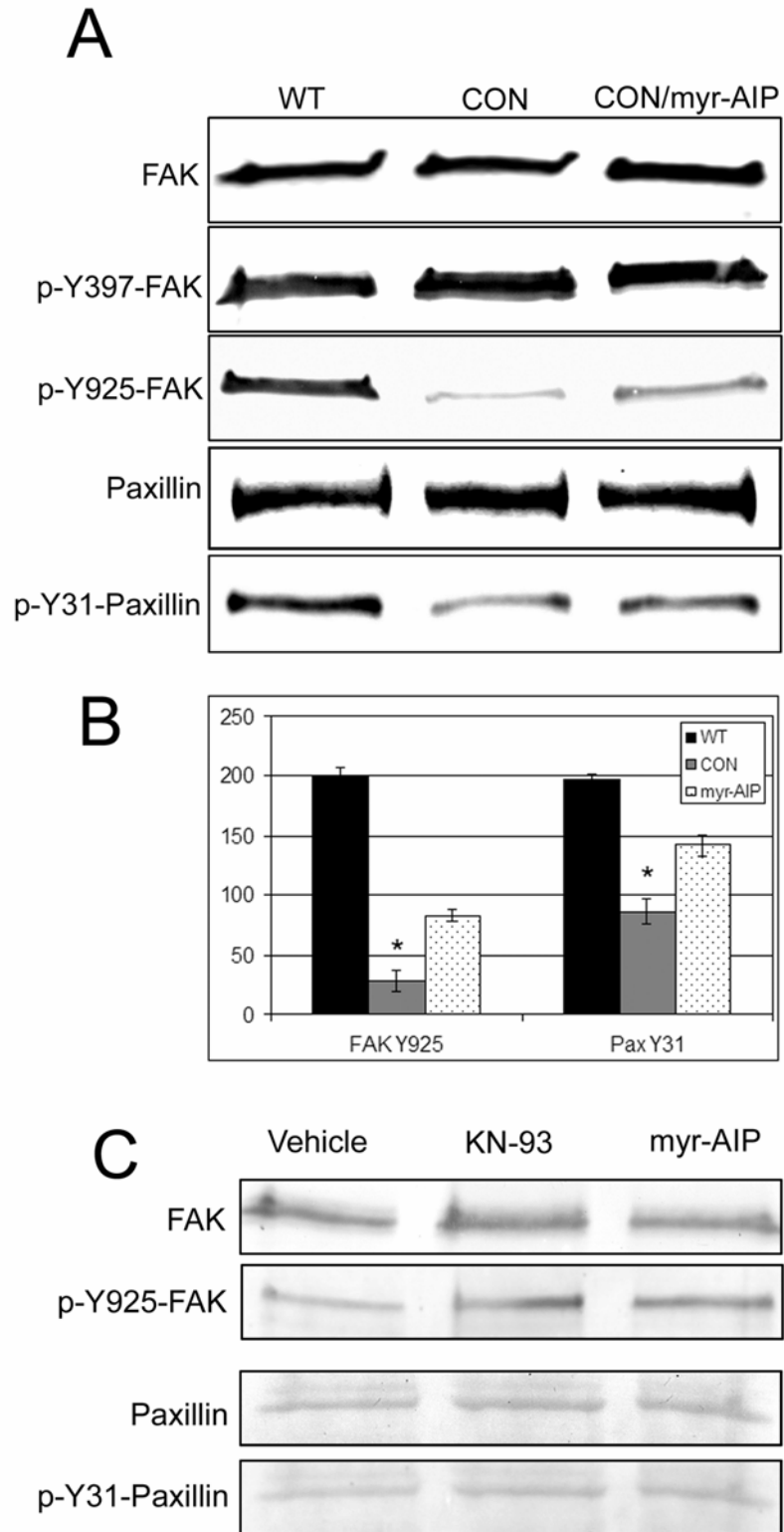
**Figure 10. Inhibition of endogenous CaMK-II enlarges paxillin-containing complexes.**

NIH/3T3 cells were transfected with GFP-paxillin, sub-cultured on 1  $\mu\text{g/ml}$  FN for 3 hours in the presence of vehicle alone (A) or 20  $\mu\text{M}$  myr-AIP (B) and imaged with traditional fluorescence microscopy. Scale bar, 50  $\mu\text{m}$ . (C) The area of each GFP-paxillin adhesion (from >10 cells and > 600 adhesions for each condition) was calculated. The minimum area for focal adhesions was 1.7  $\mu\text{m}^2$ , and maximum was 35  $\mu\text{m}^2$ . (D) Graphical representation of average focal adhesion size of NIH/3T3 cells transfected with either GFP-labeled paxillin, FAK, vinculin or talin and sub-cultured on 1  $\mu\text{g/ml}$  FN for 3 hours in the presence or absence of 20  $\mu\text{M}$  myr-AIP.



**Figure 11. Constitutively active CaMK-II decreases phospho-tyrosine levels on FAK and paxillin.** (A) NIH/3T3 cells were co-transfected with GFP-FAK or GFP-paxillin and either wild-type (WT)  $\delta_C$  CaMK-II or constitutively active (CON)  $\delta_C$  CaMK-II and sub-cultured on 1  $\mu\text{g/ml}$  FN for 3 hours. Cells expressing CON  $\delta_C$  CaMK-II/GFP-FAK or GFP-paxillin were treated with vehicle alone or with 20  $\mu\text{M}$  myr-AIP during sub-culture (as indicated). GFP-FAK and GFP-paxillin lysates were then probed with the indicated phospho or total antibody. (B) Densitometry from 3 different immunoblots for FAK Y925 and paxillin Y31. (C) NIH/3T3 cells were sub-cultured on 1  $\mu\text{g/ml}$  FN for 3 hours in the presence of either vehicle control, 10  $\mu\text{M}$  KN-93 or 20  $\mu\text{M}$  myr-AIP. Cells were harvested and probed with the indicated antibodies.





## CHAPTER 3: Protein Targets Downstream of CaMK-II Activity

The results presented thus far provide the first direct evidence of a link between CaMK-II activity and focal adhesion turnover as a means of influencing cell adhesion and motility. Previous studies have shown that CaMK-II influences de-adhesion by disrupting  $\beta 1$  integrin clustering through either direct phosphorylation of  $\beta 1$  integrin (Takahashi 2001; Wennerberg et al. 1998) or phosphorylation of integrin cytoplasmic associated protein-1 alpha, ICAP-1 $\alpha$  (Blystone et al. 1999). However, many cell types, including NIH/3T3 embryonic fibroblasts, lack ICAP-1 $\alpha$  (Bouvard et al. 2003). Furthermore,  $\beta 1$  integrin engagement, which induces FAK autophosphorylation (Mitra et al. 2005), was not affected by CON CaMK-II expression or CaMK-II inhibition. Our results are consistent with previous findings implicating  $\text{Ca}^{2+}$  transients with focal adhesion disassembly (Conklin et al. 2005) and reveal a novel means by which CaMK-II influences focal adhesion turnover by transiently promoting the tyrosine dephosphorylation of both FAK and paxillin.

While we have demonstrated a unique role for CaMK-II in focal adhesion turnover, the relevant substrate has not been determined. Although CaMK-II has been implicated in the phosphorylation of FAK on S<sup>843</sup> within minutes of G-protein receptor activation (Fan et al. 2005), the phosphorylation status of FAK S<sup>843</sup> was not altered upon CaMK-II overexpression or inhibition. Therefore it is not likely that CaMK-II directly

phosphorylates FAK under the conditions described here. Rather, the reduction in FAK and paxillin phospho-tyrosine levels caused by constitutively active CaMK-II is most consistent with the activation of a tyrosine phosphatase.

The most likely candidate tyrosine phosphatase downstream of CaMK-II activity is the SH2 domain-containing protein tyrosine phosphatase 2 (SHP-2), also known as PTPN11 or PTP1D. CaMK-II has been implicated upstream from SHP-2 in T-cell signaling (McGargill et al. 2005), but this link has not been investigated with respect to cell motility and/or focal adhesion turnover. SHP-2, a non-receptor tyrosine phosphatase, translocates from the ER to focal adhesions in response to src signaling (Wang et al. 2006) to dephosphorylate FAK on Y<sup>925</sup> and paxillin on Y<sup>31</sup>, thus promoting detachment from the extracellular matrix (Vadlamudi et al. 2002). Like CaMK-II inhibition, the knockout or inhibition of SHP-2 increases focal adhesion size and blocks cell motility in both cells in culture and in mouse embryos (Inagaki et al. 2000; MacGillivray et al. 2003; Manes et al. 1999; Saxton et al. 2000; Wang et al. 2005; Yu et al. 1998). While SHP-2 activity is known to be regulated by tyrosine phosphorylation, this enzyme can also be phosphorylated on serine/threonine residues (Poole and Jones 2005).

In this study, we evaluated potential CaMK-II substrates involved in focal adhesion dynamics. Based on previous results and sequence analysis, we tested the ability of CaMK-II to phosphorylate 8 different proteins found to influence focal adhesions. To further link SHP-2 in a novel pathway downstream of CaMK-II activation, we used dominant/negative and constitutively active mutant SHP-2 constructs in conjunction with constitutively active CaMK-II to visualize effects on paxillin-containing focal adhesions. The results of this

study suggest that CaMK-II elevates SHP-2 phosphatase activity by inducing tyrosine phosphorylation of Gab1, a protein that activates SHP-2.

## Materials and Methods

*NIH/3T3 culture and harvesting-* NIH/3T3 cells were cultured on tissue culture dishes (Nunc, Rochester NY) at 37°C in DMEM with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad CA). Cells were sub-cultured every 3 to 4 days, never exceeding 95% confluency. When specified, dishes were pre-incubated with 1 µg/ml human fibronectin (Invitrogen) in PBS (Phosphate Buffered Saline) for 1 hour at 37°C or overnight at 4°C, washed once with PBS and then placed into DMEM/10% FBS just prior to plating cells. Cells were harvested by trypsinization, washed in ice-cold PBS and then resuspended in homogenization buffer, which consisted of 30 mM Hepes pH 7.4, 20 mM MgCl<sub>2</sub>, 80 mM β-glycerol phosphate, 2.6 mM EGTA, 0.1 µM okadaic acid, 1 µg/ml each chymostatin, leupeptin, antipain, pepstatin and soybean trypsin inhibitor. Cells were lysed using two four-second bursts from a probe sonicator (Misonix, Farmingdale NY) and then centrifuged at 12,000 x g for 15 minutes at 4°C.

*Plasmid Constructs-* EGFP-linked CaMK-II constructs used in this study were prepared as previously described (Lantsman and Tombes 2005). EGFP-paxillin was prepared as described (Brown et al. 2006; Webb et al. 2004). Wild-type SHP-2, dominant/negative SHP-2 and constitutively active SHP-2 were all obtained from Addgene (Plasmid numbers 8381, 8382 and 8321, respectively). EGFP-talin and vinculin were generous gifts from Dr.

Kenneth Yamada, National Institutes of Health, Bethesda MD and Dr. Benjamin Geiger, Weizmann Institute of Science, Rehovot Israel, respectively.

*Transfection and Microscopy*- Freshly sub-cultured cells were transfected with Lipofectamine 2000 as specified (Invitrogen). Live or formaldehyde fixed cells were imaged in phase contrast or traditional fluorescence (Fm) using an IX-70 inverted microscope equipped with a 12-bit black/white F-View CCD camera and processed using Microsuite-B3SV Version 3.2 software (Olympus, Melville NY). Confocal Images were acquired with an IX-81 inverted Spinning Disk Confocal microscope equipped with an Orcaer monochrome CCD camera and processed using SlideBook Version 4.2 (Olympus).

*Immunoprecipitation and Western Blotting*- Transfected NIH/3T3 cells were washed once with ice-cold PBS and then lysed with a minimal volume of homogenization buffer (see *Cell Culture and Harvesting*) containing 0.1% NP-40 (Pierce) for 30 minutes on ice. Cell lysates were scraped into microcentrifuge tubes and sonicated for 2 seconds. Lysates were then centrifuged at 12,000 x g for 15 minutes at 4°C. GFP-tagged FAK was immunoprecipitated from 500 µg of total lysate protein for 16 hours at 4°C. Immuno-complexes were then incubated for 2 hours with protein G magnetic beads (DynaBeads, Carlsbad, CA). Magnetic beads were washed 3 times and resuspended in 2X SDS loading buffer with DTT. Proteins were resolved on 10% polyacrylamide gels and then transferred to nitrocellulose. Immunoblots were blocked with 5% BSA, TBSTA containing 5% goat

serum. Blots were then incubated overnight with 1  $\mu\text{g/ml}$  primary antibody in 5% BSA, TBSTA. After a 2 hour incubation with 2  $\mu\text{g/ml}$  alkaline phosphatase conjugated secondary antibodies, blots were developed with 0.25 mg/ml BCIP/NBT (Roche, Indiannapolis IN) in phosphatase buffer (Lantsman and Tombes 2005).

*CaMK-II Phosphorylation Assay*- Immunoprecipitated focal adhesion proteins were incubated alone, with purified CaMK-II or with CaMK-II and myr-AIP for 30 minutes at 32°C in buffer containing 30 mM HEPES (pH 7.4), 0.1 mM dithiothreitol, 15 mM magnesium acetate, 50 mM  $\beta$ -glycerophosphate, 0.5 mM cyclic AMP dependent protein kinase inhibitor peptide, 0.5 mCi of [ $\gamma$ - $^{32}\text{P}$ ] ATP, 20 mM ATP, 1 mM calmodulin, and 1 mM EGTA/3 mM  $\text{CaCl}_2$ . Phosphorylation reactions were stopped with an equal volume of preheated 2X sample buffer, followed by boiling for 3 minutes. Samples were then subjected to SDS-PAGE and transferred to nitrocellulose. Transferred proteins were then exposed to X-Blue film (Kodak, Rochester, NY) at -80°C.

*Reagents*- The GFP immunoprecipitating antibody was from Invitrogen (Carlsbad, CA). Immunoprecipitating antibodies for paxillin, FAK,  $\beta 1$  integrin and SHP-2 were all from BD Biosciences (San Jose, CA). The Gab1 immunoprecipitating antibody was from Cell Signaling (Danvers, MA). Myr-AIP, a peptide mimetic for the autoinhibitory domain of CaMK-II, was from Biomol (Plymouth Meeting, PA). Radio-labeled  $\gamma$ -ATP was from Perkin Elmer (Waltham, MA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

## Results

***Myr-AIP Treatment Cannot Recover CON CaMK-II-induced Cell Rounding in Cells Expressing SHP-2 CON.*** Because expression of CON CaMK-II induced tyrosine dephosphorylation of sites on FAK and paxillin, we evaluated whether the SH2-domain containing tyrosine phosphatase 2 (SHP-2) lies downstream of CaMK-II activity. To assess the effects of constitutively active SHP-2 (SHP-2 CON) on cell morphology and paxillin-containing focal adhesions, NIH/3T3 cells were transfected with GFP-tagged paxillin, unlabeled CON CaMK-II and either empty vector or unlabeled SHP-2 CON. 24 hours after transfection, cells were sub-cultured on 1µg/ml FN for three hours, formaldehyde-fixed and imaged under spinning disk confocal microscopy (Figure 12). As previously shown, cells expressing CON CaMK-II failed to spread on FN and showed a near elimination of paxillin-containing focal adhesions. This phenotype was recovered when CON CaMK-II expressing cells were cultured in the presence of 20 µM myr-AIP (Figure 12). Similarly, cells expressing both CON CaMK-II and SHP-2 CON showed a rounded cell morphology and a loss of paxillin-containing focal adhesions. However, focal adhesions and cell spreading could not be recovered in cells expressing both CON CaMK-II and SHP-2 CON (Figure 12). These results suggest that SHP-2 CON induces effects on cell morphology and focal adhesions similar to that of CON CaMK-II and that myr-AIP cannot recover the SHP-2 CON mutant phenotype.



***Expression of Dominant/negative SHP-2 Partially Reverses the CON CaMK-II***

***Phenotype.*** To further demonstrate that SHP-2 lies downstream of CaMK-II, NIH/3T3 cells were transfected with GFP-tagged paxillin, unlabeled CON CaMK-II and either empty vector or dominant/negative SHP-2 (D/N SHP-2). 24 hours after transfection, cells were plated on FN for 3 hours, fixed and imaged using traditional fluorescence microscopy (Figure 13). Cells expressing CON CaMK-II/empty vector were rounded and showed a loss of paxillin-containing focal adhesions (Figure 13). Cells expressing D/N SHP-2 still retained a more rounded cell morphology, but these cells exhibited a strong increase paxillin-containing focal adhesions at the periphery of the cell (Figure 13). These results suggest that SHP-2 lies downstream of CaMK-II activation in causing focal adhesion turnover.

***CaMK-II Does Not Phosphorylate SHP-2 in vitro.*** Previous results suggested that CaMK-II might influence SHP-2 activity directly. To determine whether CaMK-II could phosphorylate SHP-2, immunopurified SHP-2 was used as a substrate in a CaMK-II phosphorylation assay. Sequence analysis of SHP-2 reveals 10 CaMK-II consensus sequences (Figure 14). To test whether these sites are phosphorylated in native SHP-2, immunoprecipitated SHP-2 (from NIH/3T3 cells) was incubated with purified CaMK-II for 30 minutes at 32°C in CaMK-II kinase specific buffer. As a control, SHP-2 was incubated without CaMK-II, and to show specificity, a third condition contained SHP-2 and CaMK-II incubated with 50 µM myr-AIP. Table 1 shows the results of several

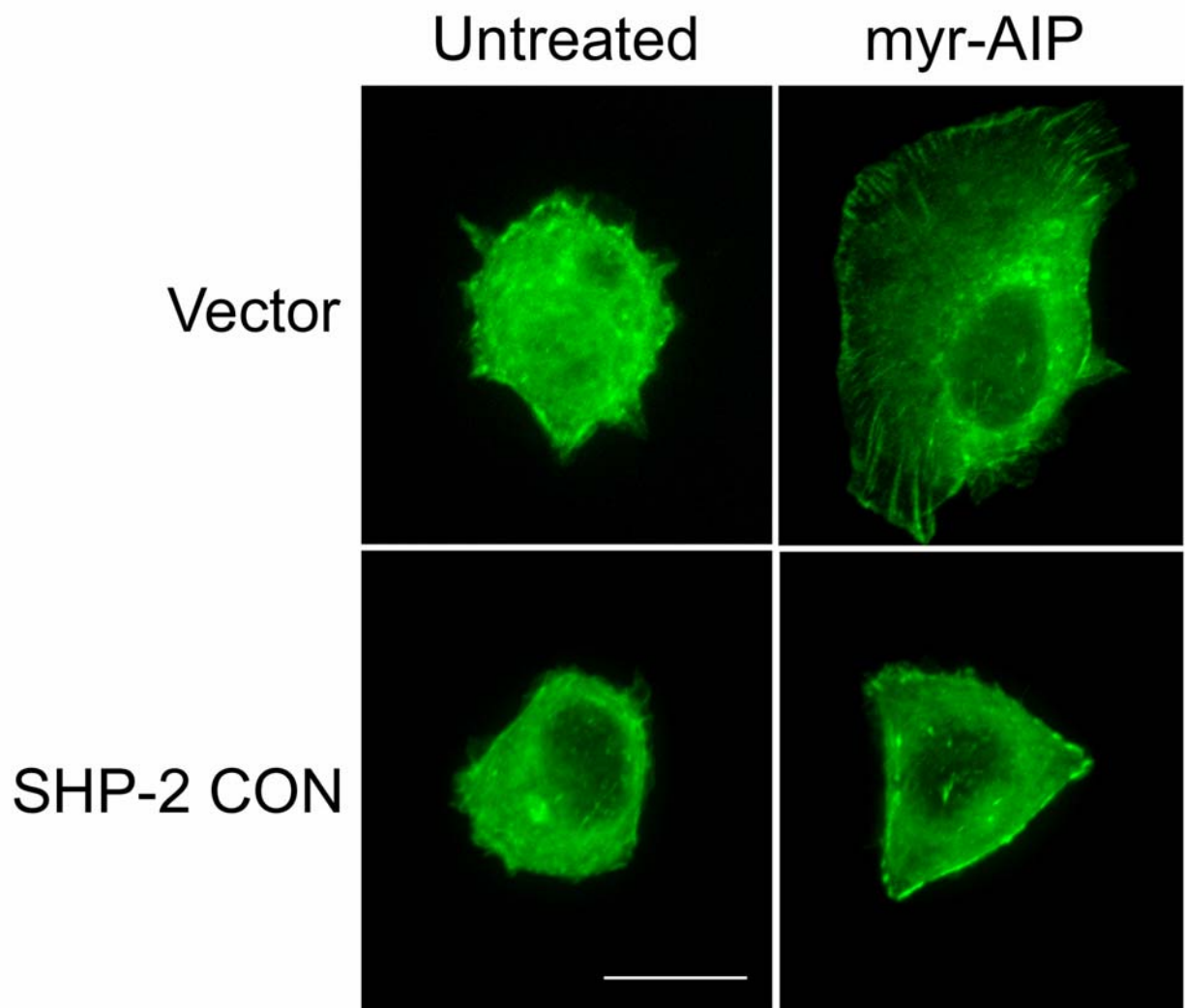
different trials with 8 different focal adhesion proteins, including SHP-2. These results suggest that CaMK-II was unable to phosphorylate SHP-2 and other focal adhesion proteins *in vitro*. Our results showing an inability to phosphorylate SHP-2 with CaMK-II are similar to difficulties in other studies implicating SHP-2 downstream of CaMK-II (McGargill et al. 2005).

***CON CaMK-II Expression Increases Tyrosine Phosphorylation of Gab1 on Y<sup>627</sup>.***

Because CaMK-II does not appear to affect SHP-2 activity directly, we next evaluated whether CaMK-II influenced the tyrosine phosphorylation levels of Gab1, a protein known to bind SHP-2 and induce its activation (Cunnick et al. 2001). Specifically, Y<sup>627</sup> is a critical phosphorylation site necessary for SHP-2 activation. This residue can be phosphorylated by src family kinases, but also possibly c-abl, as Gab1 recruits c-abl to the plasma membrane (Holgado-Madruga et al. 1996). To evaluate Gab1 phosphorylation, cells were transfected with either GFP-WT CaMK-II or GFP-CON CaMK-II. 24 hours post transfection, cells were sub-cultured on FN with or without 20  $\mu$ M myr-AIP as indicated for three hours and harvested. Lysates were then probed with phospho-Y<sup>627</sup> and total Gab1 antibodies. Cells expressing CON CaMK-II exhibited a significant increase in Gab Y<sup>627</sup> phosphorylation as compared to WT expressing cells, and this increase was diminished when CON CaMK-II expressing cells were sub-cultured in the presence of myr-AIP (Figure 15). While CaMK-II cannot phosphorylate Gab1 directly (Table 1), these results indicate that CaMK-II influences SHP-2 activation by stimulating Gab1 tyrosine phosphorylation through an unknown mechanism.

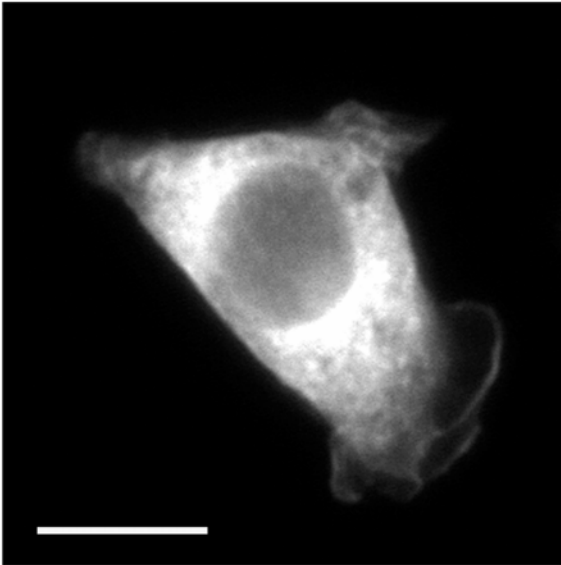
***c-abl Inhibition Rescues CON CaMK-II-induced Cell Rounding and Focal Adhesion Disassembly.*** As previously mentioned, c-abl is a tyrosine kinase involved in cell motility and binds to Gab1 (Hernandez et al. 2004; Holgado-Madruga et al. 1996). c-abl is activated by tyrosine phosphorylation by src family kinases, but may be regulated by serine/threonine phosphorylation as well (Hernandez et al. 2004) (and unpublished data, see Cell Signaling c-abl product information). To determine whether c-abl is downstream of CaMK-II, NIH/3T3 cells were transfected with GFP-tagged paxillin and either unlabeled WT or CON CaMK-II. 24 hours after transfection, cells were sub-cultured on FN for 3 hours, fixed and imaged using traditional fluorescence microscopy. CON CaMK-II expressing cells were also sub-cultured in the presence of 20  $\mu$ M myr-AIP (CON/myr-AIP) or 10  $\mu$ M AG957, a specific c-abl inhibitor also known as tyrphostin (CON/AG957). As previously shown, expression of CON CaMK-II induced cell rounding and disrupted paxillin-containing focal adhesion formation (Figure 16). Like myr-AIP treatment, inhibition of c-abl recovered the CON CaMK-II phenotype by inducing cell spreading on FN and re-incorporation of paxillin into focal adhesions (Figure 16). These results strongly suggest that c-abl lies downstream of CaMK-II and provide a framework for the mechanism by which CaMK-II induces focal adhesion turnover.

**Figure 12. CaMK-II Inhibition Does Not Rescue SHP-2-mediated Focal Adhesion Disassembly.** NIH/3T3 cells were transfected with GFP-paxillin, constitutively active (CON) CaMK-II, and either vector alone or constitutively active SHP-2 (SHP-2-CON), as indicated. Following sub-culture on 1  $\mu$ g/ml FN for 3 hours with or without 20  $\mu$ M myr-AIP, cells were fixed and imaged under spinning disk confocal microscopy. Scale bar, 50  $\mu$ m.

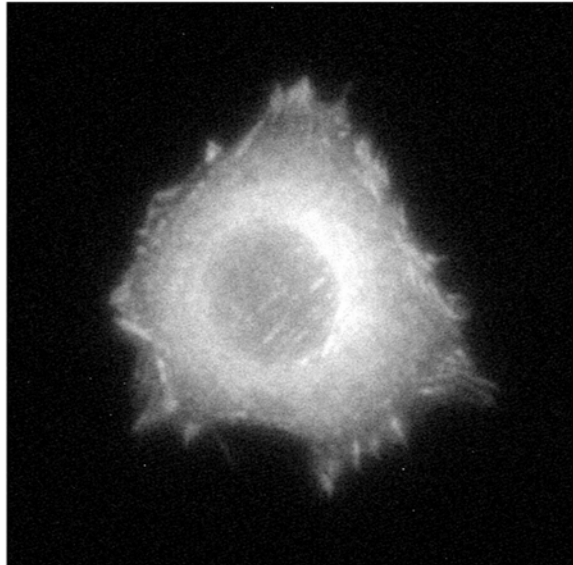


**Figure 13. D/N SHP-2 Expression Partially Reverses CON CaMK-II-mediated Cell Rounding.** NIH/3T3 cells were transfected with GFP-paxillin, constitutively active (CON) CaMK-II, and either vector alone or dominant/negative SHP-2 (D/N SHP-2), as indicated. Following sub-culture on FN for 3 hours cells were fixed and imaged under traditional fluorescence microscopy. Scale bar, 50  $\mu$ m.

Vector



D/N SHP-2



**Figure 14. SHP-2 Contains Several CaMK-II Consensus Sequences.** Sequence analysis of SHP-2 reveals a number of potential CaMK-II phosphorylation sites.



**SHP-2 Human Sequence : 10 CaMK-II consensus phosphorylation sequences T73, T127, T134, S160, S189, S234, S365, T411, S448, S576**

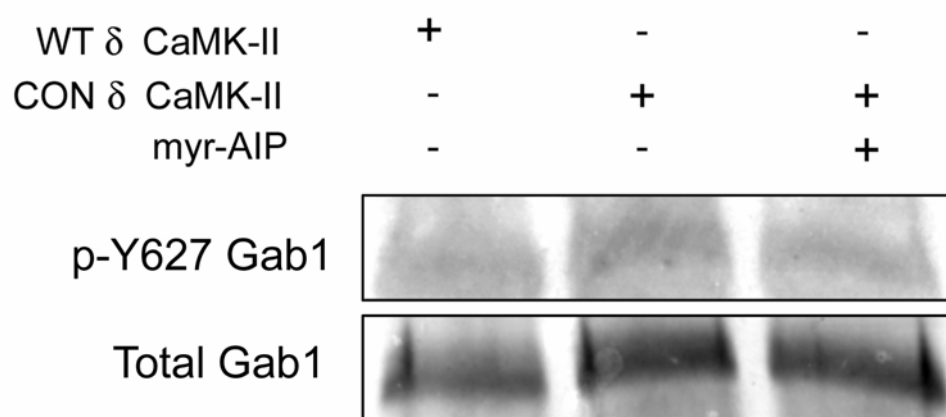
MKSRRWFHPN	ITGVEAENLL	LTRGVDGSFL	ARPSKSNPGD	LTLSVRRNGA
VTHIKIQNTG	DYYDLYGGEK	<b>FAT</b> LAEVLQY	YMEHHGQLKE	KNGDVIELKY
PLNCADPTSE	RWFHGHLSGK	EAE <b>KLLT</b> EGK	<b>KHGS</b> FLVRES	QSHPGDFVLS
VRTGDD <b>KGES</b>	NDGKSKVTHV	MIRCQELKYD	VGGGER <b>FDSL</b>	TDLVEHYKKN
PMVETLGTVL	QLKQPLNTTR	INAAEIESRV	<b>RELS</b> KLAETT	DKVKQGFWE
FETLQQQECK	LLYSRKEGQR	QENKNKNRYK	NILPFDHTRV	VLHDGDPNEP
VSDYINANII	MPEFETKCNN	SKPKKSYIAT	QGCLQNTVND	FWRMVFQENS
RVIVMTTKEV	<b>ERGKS</b> KCVKY	WPDEYALKEY	GVMRVRNVKE	SAAHDYTLRE
LKLSKVG <b>QGN</b>	<b>TERT</b> VWQYHF	RTWPDHGVPS	DPGGVLDLFLE	EVHH <b>KQES</b> IM
DAGPVVVHCS	AGIGRTGTFI	VIDILIDIIR	EKGVDCDIDV	PKTIQMVRSQ
RSGMVQTEAQ	YRSIYMAVQH	YIETLQRRIE	EEQKSKRKGH	EYTNIKYSLA
DQTSGDQSPL	PPCTPTPPCA	EM <b>REDS</b> ARVY	ENVGLMQQQK	SFR

**Table 1. Analysis of Potential CaMK-II Targets Involved in Focal Adhesion Dynamics.** Each target was evaluated by sequence analysis and by CaMK-II *in vitro* phosphorylation assay. Where applicable, citations are listed to indicate previous research implicating specific proteins as CaMK-II targets.

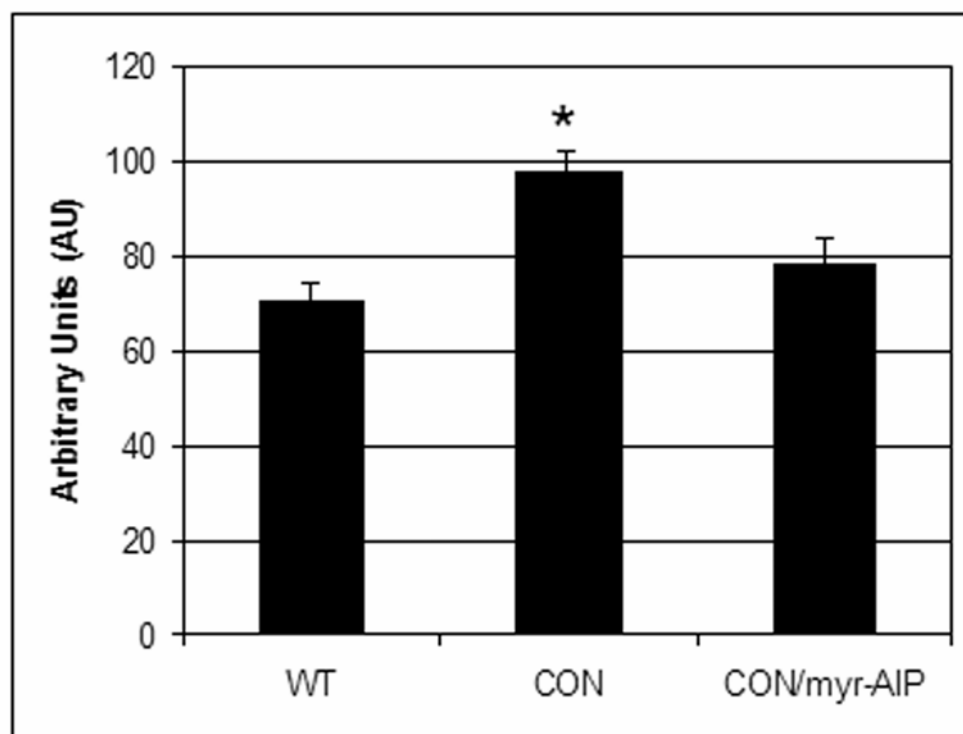
Target Proteins	CaMK-II Able to Phosphorylate?	Evidence For or Against Phosphorylation
Paxillin	No	No CaMK-II consensus sequences
FAK	Yes, Ser843 (Fan et. al. 2005)	Yes, but p-Ser843 levels are unchanged in our experimental approach
Vinculin	No	No CaMK-II consensus sequences
Talin	No	No CaMK-II consensus sequences
$\beta$ 1 Integrin	Yes, Ser789 (Takahashi et. al 2001 and Wennerberg et. al. 1998)	Yes, but not in our <i>in vitro</i> assays, and when phosphorylated, blocks FAK autophosphorylation and talin recruitment to focal adhesions
SHP-2	No	Contains CaMK-II consensus sequences, but cannot be phosphorylated <i>in vitro</i> by our lab or McGargill et. al. 2005
Gab1	No	No real CaMK-II consensus sequences
c-abl	Potentially	Contains CaMK-II consensus sequences

**Figure 15. Constitutively active CaMK-II increases the tyrosine phosphorylation of Gab1.** **A.** NIH/3T3 cells were transfected with either wild-type (WT) GFP-CaMK-II or constitutively active (CON) GFP-CaMK-II and sub-cultured on 1  $\mu$ g/ml FN for 3 hours. Cells expressing CON GFP-CaMK-II were treated with vehicle alone or with 20  $\mu$ M myr-AIP during sub-culture (as indicated). Gab1 was then immunoprecipitated from transfected lysates and probed with either phospho-Y<sup>627</sup> Gab1 or total Gab1. **B.** Densitometry of p-Y<sup>627</sup> Gab1 from 3 separate experiments, \*  $p < 0.05$ .

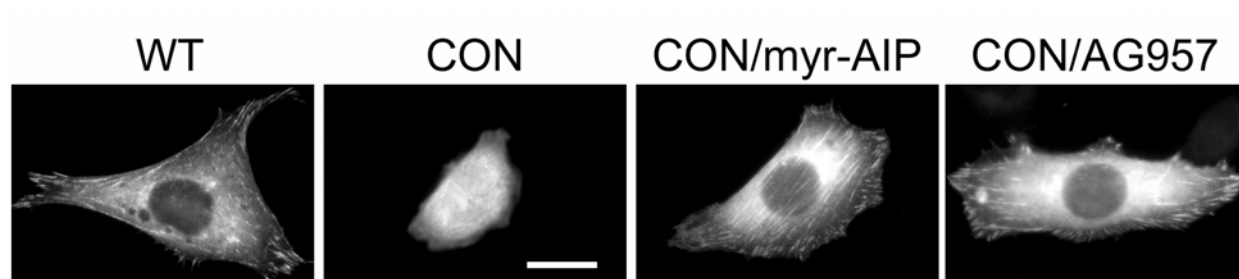
A



B

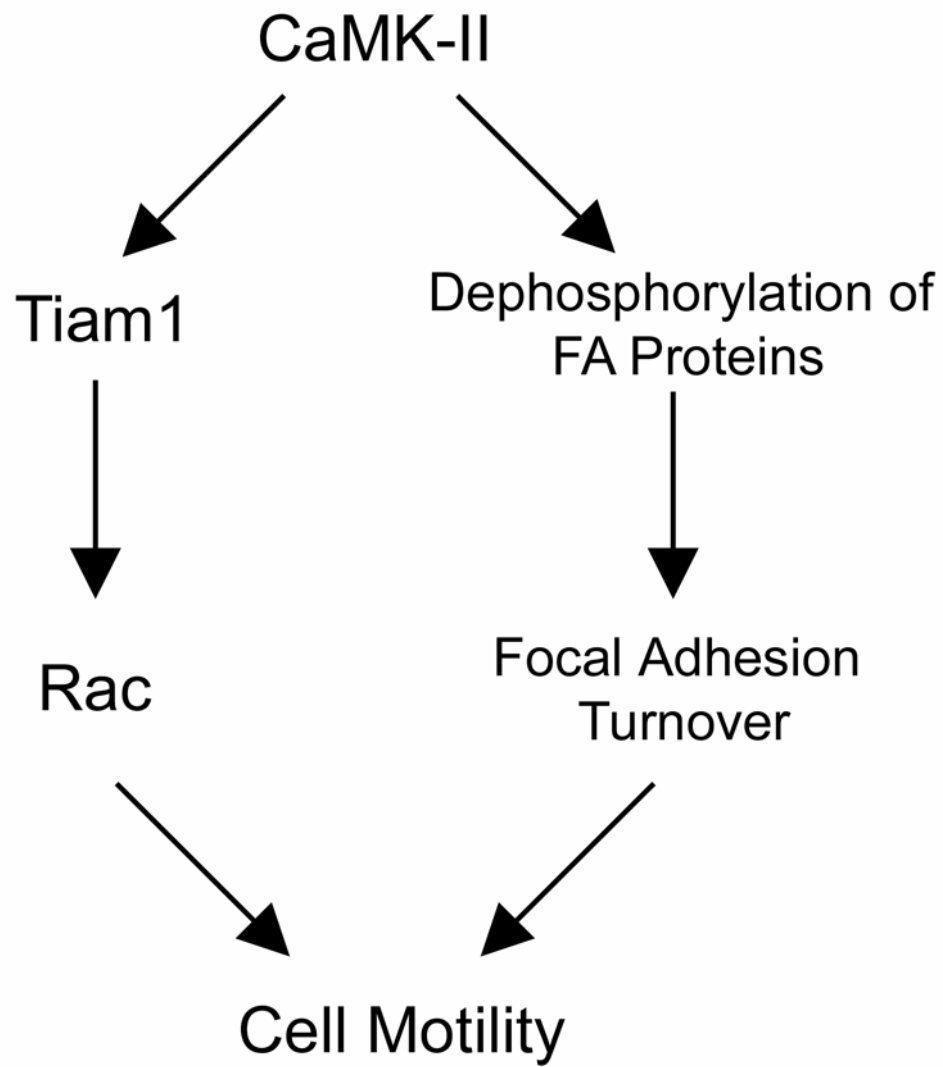


**Figure 16. c-abl Inhibition Recovers Both Cell Spreading and Paxillin-containing Focal Adhesions in Cells Expressing CON CaMK-II.** NIH/3T3 cells were transfected with GFP-tagged paxillin, and either wild type (WT) or constitutively active (CON) CaMK-II. Cells were sub-cultured on FN for 3 hours either without treatment or in the presence of 20  $\mu$ M myr-AIP (CON/myr-AIP) or 10  $\mu$ M AG957 (CON/AG957). Cells were fixed and imaged under traditional fluorescence microscopy. Scale bar, 50  $\mu$ m.

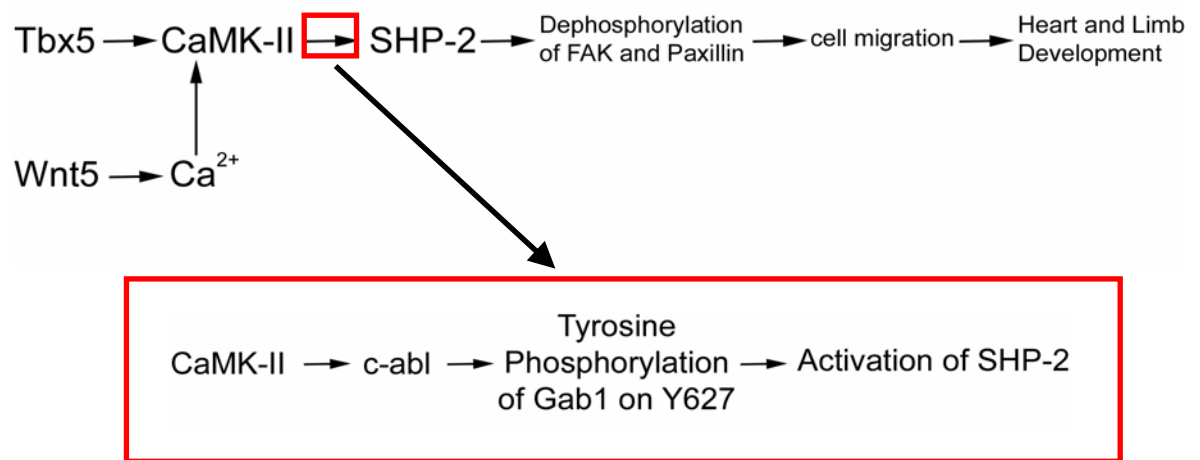


**Figure 17. CaMK-II Enables Cell Motility By Activating Tiam1 and Inducing Focal Adhesion Turnover.** Schematic depicting the dual role for CaMK-II in regulating cell motility.





**Figure 18. A Novel Role for CaMK-II Linking Tyrosine Dephosphorylation of FAK and Paxillin with Heart and Limb Development.** This pathway links CaMK-II expression and activation through the non-canonical Wnt pathway with focal adhesion turnover in promoting proper heart and limb development.



## General Discussion

### *Dual Role for CaMK-II in Cell Motility*

While the results presented here implicate CaMK-II in a novel pathway regulating focal adhesion turnover, CaMK-II has been previously linked to cell motility (Bilato et al. 1997; Bouvard and Block 1998; Bouvard et al. 1998; Lundberg et al. 1998; Pauly et al. 1995; Pfeleiderer et al. 2004; Suzuki and Takahashi 2003). More recently, CaMK-II has been shown to phosphorylate and activate Tiam1 (tumor invasion and metastasis 1), a RhoGEF that activates Rac (Fleming et al. 1999; Hamelers et al. 2005). Rac then induces actin polymerization and lamellipodial extension at the leading edge of the cell to promote cell motility. However, expression of constitutively active Rac enhances both cell spreading on FN and cell motility (Kerr et al. 2008). This phenotype is the exact opposite of CON CaMK-II expression which leads to decreased cell adhesion (by inducing focal adhesion turnover) and decreased cell motility. Thus CaMK-II appears to have a dual role in regulating cell motility (Figure 17), in which CaMK-II promotes cell motility by activating TIAM1 to drive lamellipodial extension and by activating SHP-2 to induce focal adhesion turnover.

Like CaMK-II both SHP-2 and FAK are critically involved in focal adhesion dynamics in that inhibition or knockout of FAK or SHP-2 increase focal adhesion size and block cell motility (Ilic et al. 1995; Inagaki et al. 2000; MacGillivray et al. 2003; Manes et al. 1999; Saxton et al. 2000; Wang et al. 2005; Yu et al. 1998). Similarly to CON CaMK-

II, expression constitutively active SHP-2 or FAK also disrupts cell motility (Poole and Jones 2005; von Wichert et al. 2003). Results such as these, which suggest that there are optimal activity levels for CaMK-II, SHP-2 and FAK, support common roles for each enzyme in promoting the cyclical formation and breakdown of focal adhesions. In this context and in order to ensure that focal adhesion disassembly does not occur immediately after assembly,  $\text{Ca}^{2+}$  elevations must be temporally offset from the initial adhesion event. Evidence supports such a model in that talin recruits PIPKI $\gamma$  (Di Paolo et al. 2002), which is activated by src to catalyze the synthesis of the phospholipid, PIP<sub>2</sub> (Ling et al. 2003). FAK then recruits phospholipase C (PLC $\gamma$ ) to focal adhesions (Mitra et al. 2005), which generates IP<sub>3</sub> from the patches of PIP<sub>2</sub> that accumulate over time at the cell surface (Huang et al. 2004). IP<sub>3</sub> then stimulates localized  $\text{Ca}^{2+}$  release from the ER, which resides near focal adhesions and SHP-2 (Wang et al. 2006).

The results presented here suggest that the attachment of cells to FN triggers focal adhesion assembly, which eventually yields a localized, transient  $\text{Ca}^{2+}$  release from the ER to activate CaMK-II. Activated CaMK-II then indirectly activates a protein tyrosine phosphatase SHP-2, through c-abl and Gab1, to dephosphorylate a limited number of tyrosine residues on FAK and paxillin, thus promoting focal adhesion turnover. Concurrently, CaMK-II activates TIAM1 to induce lamellipodial extension, and this process combined with focal adhesion turnover enables cell motility.

### *Role for CaMK-II in Embryonic Development*

During early embryonic development, the non-canonical *wnt* family of morphogens activate CaMK-II to drive convergent extension cell migration, an event that occurs during and after gastrulation (Kohn and Moon 2005; Kuhl et al. 2000). Also, non-canonical wnts have been implicated in both heart and limb development, two processes that require cell motility and proliferation (Grandel and Schulte-Merker 1998; Schoenebeck and Yelon 2007). Results presented here coupled with previous studies implicating CaMK-II in cell cycle progression (Morris et al. 1998; Tombes et al. 1995) suggest that CaMK-II is a critical signaling protein in both heart and limb development in vertebrates.

The inability of CaMK-II to phosphorylate SHP-2 *in vitro* is quite an interesting phenomenon given the fact that mutations in some of the CaMK-II consensus sites lead to severe defects in both heart and limb formation. Specifically, mutations in certain CaMK-II consensus sites lead to defects in heart septation and cause shortened stature in humans, a condition referred to as Noonan Syndrome (van der Burgt 2007). Interestingly, inhibition of CaMK-II or knockout of the heart and limb specific CaMK-II results in improper heart development and a loss of forelimbs (pectoral fins) in zebrafish (*Danio rerio*) (unpublished data, Rothschild, SC, Easley CA, et. al. Tbx5-mediated  $\beta 2$  CaMK-II Expression is Required for Heart Looping and Pectoral Fin Development). Like CaMK-II, knockout or inactivation of SHP-2 causes pericardial sac enlargement, defects in cardiac looping and foreshortened pectoral fins in zebrafish (Jopling et al. 2007; Razzaque et al. 2007). These similarities in morphant phenotype link CaMK-II and SHP-2 in a novel pathway in which

Tbx5-mediated CaMK-II expression, activated by non-canonical wnts, enables cell motility by inducing focal adhesion turnover via SHP-2 activation (Figure 18).

#### *Other Potential Pathways Influenced by CaMK-II*

Along with promoting cell motility, integrin engagement with FN promotes cell survival and cell cycle progression through the activation of FAK which in turn elevates ERK1/2 levels and activates PI3-K (Walker and Assoian 2005). PI3-K is known to promote cell growth, viability and migration through Akt signaling (Cantley 2002). As shown in this dissertation, inhibition of CaMK-II enhances cell adhesion, an event that should elevate levels of phospho-Akt and phospho-ERK1/2. However, cells treated with KN-93 for 24 hours show a significant reduction in both Akt and ERK1/2 phosphorylation levels (Appendix C) as determined using BioRad's Bioplex system. As mentioned, KN-93-mediated CaMK-II inhibition blocks cell cycle progression by reducing cyclin D levels, and this result is similar to those seen by PI3-K inhibition (Cantley 2002). Currently, the mechanism by which CaMK-II influences cyclin D expression is unknown, yet the results from the Bioplex Assay suggest CaMK-II promotes cell cycle progression through PI3-K signaling. Further studies using CON CaMK-II will be conducted to assess the influence of CaMK-II activity on PI-3K activation and signaling.

Another interesting aspect of CaMK-II that pertains to PI-3K signaling is the localization of CaMK-II at the cell surface as seen using TIRFm. The major CaMK-II

variant expressed in NIH/3T3 cells (and the variant used throughout this dissertation) is the simplest form of CaMK-II and contains no domains that would link CaMK-II to the plasma membrane. To localize to the cell surface, CaMK-II may bind to Gab1 as reciprocal immunoprecipitation studies involving Gab1 and CaMK-II suggest that these proteins interact with each other (Appendix D). However, mass spectrometry analysis of CaMK-II direct binding partners did not yield Gab1 as a primary interacting protein. Thus Gab1, which recruits PI3-K, PLC $\gamma$  and SHP-2 to focal adhesions, interacts with CaMK-II through an unknown adapter protein. Identifying this protein would allow us to explore how CaMK-II is targeted to focal adhesions and might allow us to further delineate a role for CaMK-II in focal adhesion turnover. Thus from these results, we can suggest that CaMK-II is a critical signaling hub that resides at the cell surface to influence cell motility and also transduces signals through PI3-K, potentially, to regulate both cell cycle progression and cell survival.



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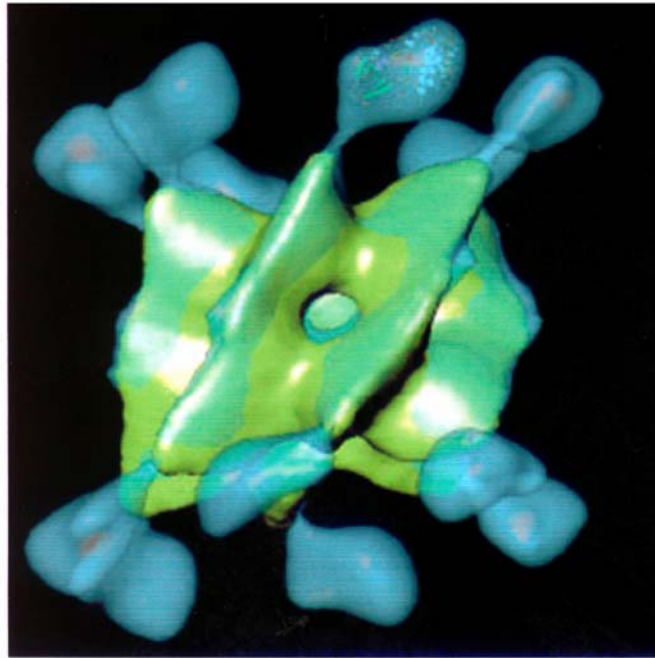


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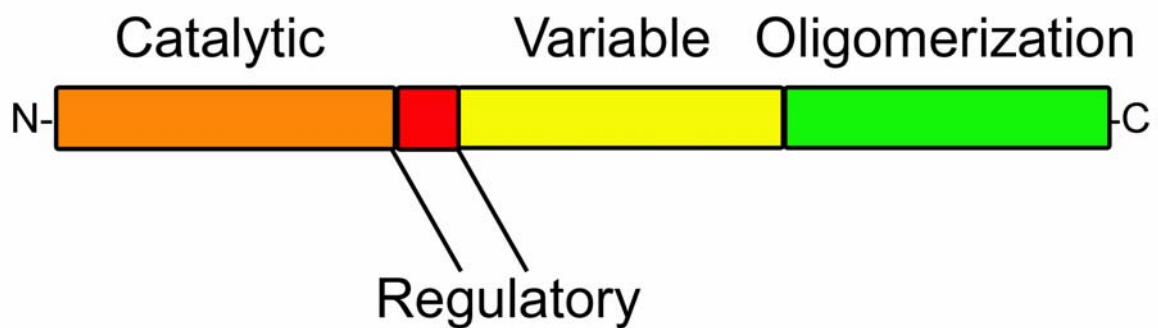
## **APPENDIX A**

**Supplemental Figure 1. CaMK-II Structure.** *A.* Cryo-electron micrograph of CaMK-II.  
*B.* Linear domain map of CaMK-II.

A



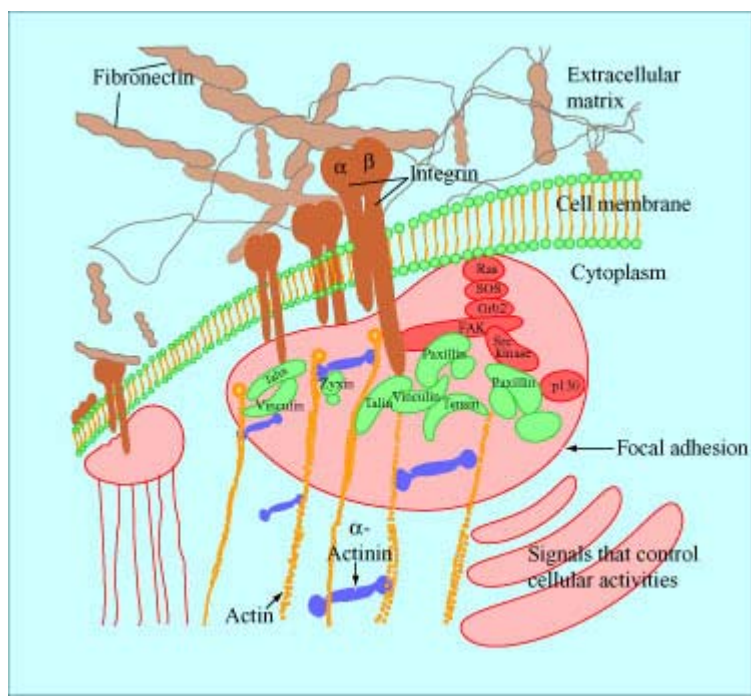
B



Part A image courtesy of Neal Waxham, Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase IIalpha and truncated CaM kinase IIalpha reveal a unique organization for its structural core and functional domains. *J. Biol. Chem.* 2000.

## **APPENDIX B**

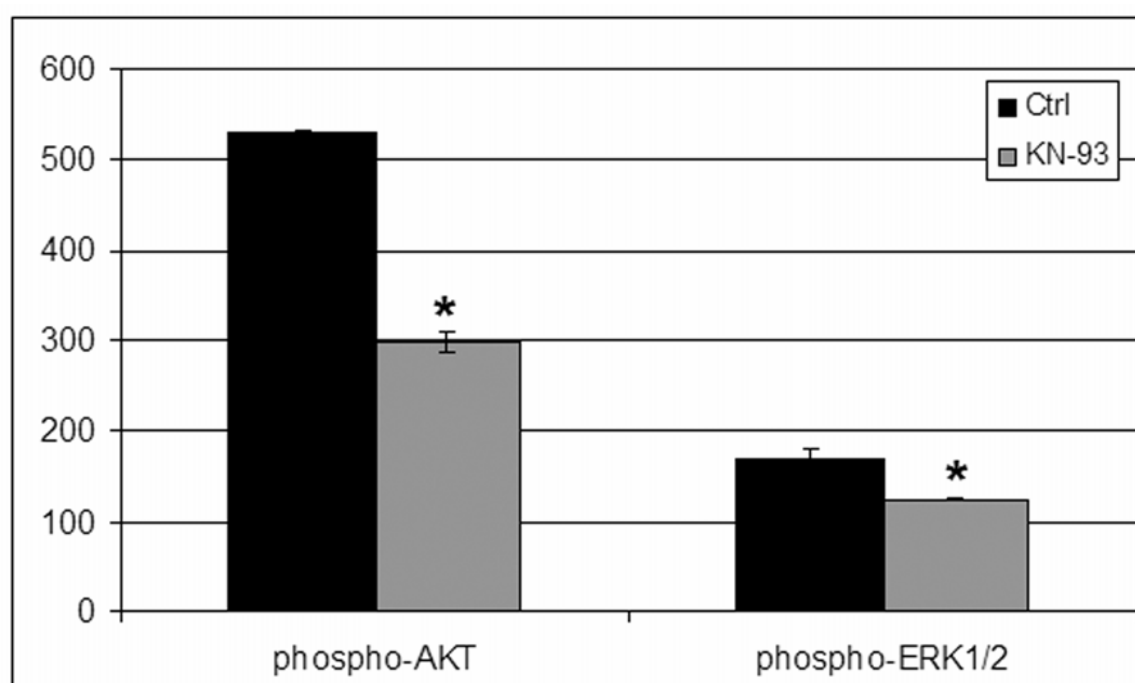
**Supplemental Figure 2. Focal Adhesion Diagram.** Schematic of a focal adhesion signaling hub.



Taken from the Massachusetts Institute of Technology Molecular, Cellular and Tissue Biomechanics course information.

### **APPENDIX C**

**Supplemental Figure 3. CaMK-II Inhibition Reduces Phosphorylation Levels of Both Akt and ERK1/2.** NIH/3T3 cells were cultured for 24 hours in the presence or absence of 10  $\mu$ M KN-93. Cells were then harvested and prepared for the BioRad Bioplex assay which measures the ratio of phospho to total protein of selected proteins, which included Akt and ERK1/2. Phospho-levels from 3 separate trials were normalized to total protein and graphed as shown. Y-axis represents arbitrary units. \* indicates statistical significance as  $p < 0.05$ .



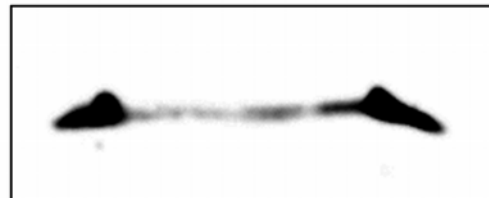
## **APPENDIX D**

**Supplemental Figure 4. CaMK-II Immunoprecipitates Contain Gab1.** *A.* Endogenous CaMK-II was immunoprecipitated from NIH/3T3 cells and probed for Gab1. Likewise, Gab1 was immunoprecipitated from cells expressing GFP-labeled CaMK-II and probed for CaMK-II. *B.* Autoradiogram of Gab1 immunoprecipitates indicating that endogenous CaMK-II associates with the Gab1. These results were all representative of 3 separate experiments.



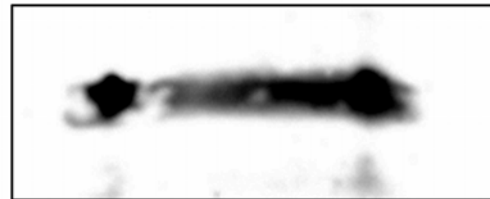
**A**

IP: Gab1



Blot: CaMK-II

IP: CaMK-II



Blot: Gab1

**B**

+ GFP-

CaMK-II

+ myr-AIP

GFP-CaMK-II →

Endogenous  
CaMK-II →

IP: Gab1

**APPENDIX E**

<b>Antibody (Company)</b>	<b>Tested Applications</b>	<b>Suitability</b>
CaMK-II (BD Biosciences)	IF, WB, IP	Worked well in all apps.
CaMK-II (Cell Signaling)	IF, WB, IP	Only worked marginally in WB
CaMK-II (Stressgen)	IF, WB, IP	Worked only in WB, very clean antibody
T286/7 CaMK-II (Millipore)	IF, WB	Works well in P19 neurons for both WB and IF, but not in 3T3 cells
T286/7 CaMK-II (Stressgen)	WB	Seems to work, but only tested once
Gab1 (Cell Signaling)	WB, IP	Worked well in all apps.
Y627 Gab1 (Cell Signaling)	WB	Weak results, would work better with GFP-Gab1
FAK (BD Biosciences)	WB, IP	Worked well in all apps.
Y397 FAK (BD Biosciences)	WB	Worked well
Y577/8 FAK (Cell Signaling)	WB	Very weak results, would not recommend
Y925 FAK (Cell Signaling)	WB	Worked well
Paxillin (BD Biosciences)	IP, WB	Worked well in all apps.
Y31 Paxillin (Biosource)	WB	Worked well
SHP-2 (BD Biosciences)	WB, IP	Worked well in all apps.
SHP-2 (Cell Signaling)	WB, IP	Works perfectly for WB. Does not IP at all.

<b>Plasmid Constructs</b>	<b>Notes</b>
FAK-GFP	Localizes well to focal adhesions and is easily detected on blots, also IP's well
Paxillin-GFP	Localizes well to focal adhesions and is detected on blots. Does not IP.
Paxillin-FLAG	Used only for IP experiments. IP's fair.
Paxillin-dsRed	Localizes well to focal adhesions. Not tested in other apps.
SHP-2-EGFP (WT, D/N)	Fluorescence is weak, but localizes normally. Can be detected by WB.
Vinculin-GFP	Localizes well to focal adhesions and is easily detected on blots. IP's well.
Talin-GFP	Localizes well to focal adhesions but is not detected on mini-blots because protein is roughly 300 kDa.

## **APPENDIX F**

*Laminin Activates CaMK-II to Stabilize Nascent Embryonic Axons*























## VITA

Charles Allen Easley, IV was born on September 21, 1979 as a citizen of the United States of America. He graduated from Woodberry Forest School in 1998. He graduated from the College of William and Mary in May 2002 with a B.S. in Biology. In August of 2005, Mr. Easley earned his M.S. in Biology from Virginia Commonwealth University and subsequently joined the Department of Biochemistry and Molecular Biology in order to obtain a Ph.D. in Biochemistry.

Mr. Easley is an active member in the American Society for Cell Biology and was recently inducted into the Phi Kappa Phi Honor Society.